EXPLORING THE EFFECTS OF CROSSLINKING ON THE INTERVERTEBRAL DISC

A Dissertation

by

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ABSTRACT

Crosslinking soft tissue has become more common in tissue engineering applications, and recent studies have demonstrated that soft tissue mechanical behavior can be directly altered through crosslinking, but increased understanding of how crosslinking affects intervertebral disc mechanical behavior is needed. In vitro testing of bovine disc and motion segments was used to characterize several important aspects of disc behavior in response to crosslinking after both soaking and injection treatment.

The first study was a comparison of different crosslinkers to determine the effect on tensile properties of disc tissue. Circumferential specimens were taken from bovine annulus and then soak treated with an optimized crosslinking formulation or sham solution. A non-contacting laser micrometer was used to measure cross sectional area, after which tension testing until failure was performed to determine yield strain, yield stress, ultimate stress, peak modulus, and resilience. The crosslinkers were observed to produce different changes in the properties, with the measured properties generally increasing

The second study used bilateral annular injections to simulate a clinically relevant delivery method. The dose response of the motion segment's neutral zone stability metrics against injection concentration was mapped. Concentrations of 20 mM and less had no significant effects on the stability metrics. 40mM demonstrated a change in

neutral zone stiffness, while at least 80mM was required to significantly affect neutral zone length. Thus, meaningful changes in joint neutral zone stability were demonstrated using clinically relevant injection and chemical formulations.

The third study used combinations of biochemical and accelerated mechanical cyclic loading to degrade gelatin and annulus fibrosus specimens with and without genipin treatment. Genipin crosslinking attenuated changes during cyclic loading to specimen geometry and compliance relative to control samples. Full recovery of genipin treated samples appeared to be hampered, at least partially from continued crosslinking during the accelerated testing.

The fourth study tested the effect of genipin crosslinking to resist interlamellar shearing of the annulus lamella. Using a recently reported test method that shears adjacent lamella, crosslinked specimens were noted to have significantly higher yield force, peak force, and resilience compared to sham treated controls, supporting the hypothesis that crosslinking would increase the load bearing ability of the interface.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1. Scope & Cost

Low back pain (LBP) is a common occurrence affecting an estimated 60-80% of the general population some time in their life [71]. Strine and Hootman [114] reported that over a 3 month period, 34 million adults in the US (17%) reported LBP. Further, LBP is not unique to the US, with such countries reporting a LBP lifetime prevalence as Thailand (7%), Nepal (18%), Sweden (31%), Yugoslovia (50%), and Finland (75%) [141].

Economically, LBP presents a significant burden. 'Back Problems' ranked as the 6th most expensive medical condition in America in 1996[33] and the National Institute of Health [114] cite back and neck problems as the leading cause of job-related disability costing the US over $50 billion each year. Outside the United States, an estimated $370 million dollars (US) was spent in the Netherlands[135] on direct medical costs to treat back pain, with an actual cost that may have been as much as $550 million dollars (US).

1.2. Understanding the Sources and Treatment of LBP

Diagnosing the source of LBP is often confounded by the absence of obvious identifiable pathologies. Fishgrund and Montgomery [37] presented the differential diagnosis for LBP as: deformity, fracture, disc (herniation or stenosis), infection, tumor, myofascial pain, intra-abdominal pain, and intrapelvic pathology. But he then noted that the 'vast majority of patients with LBP' have no deformity, tumor, infection, trauma, or

nerve-root impingement leaving the most likely source of back pain as originating in the disc or as myofascial pain. Similarly, Zhang and colleagues reported that only about 20% of LBP cases were attributed with reasonable certainty to a pathologic or anatomical entity and that discogenic lower back pain (DLBP) is the most common disease of chronic LBP accounting for 39% of incidence [151].

The underlying mechanisms that produce discogenic pain are not clear. Unlike young, healthy discs, older discs often appear degenerated and although degeneration has often been implicated as a source of pain, several studies have found that degenerated discs are not always painful discs. For example, Paajanen[94] found 35% of healthy male volunteers without back pain had significant disc degeneration on MRI and Holt reported disc degeneration in 90% of adult roentgenograms but only 47% had history of LBP[37]. Some investigators have further noted that degenerated discs often exhibit abnormal motion patterns under loading (clinical instability).

While the hypothesis of joint instability as a mechanism of disc pain has demonstrated mixed success [124], [41], [74], [70] recently Dickey [32] was able to demonstrate a strong relationship between reported pain level and motion of the spine segments. However, it is also possible that the pain associated with segmental motion pain does not arise exclusively from the disc. With disc degeneration, secondary structures such as the facet joints and para spinal ligaments may experience increased loads to compensate for

reduced disc performance. This may in turn lead to pain as the secondary structures are over stressed.

1.3. Current Treatment Options

Conservative treatment of back pain includes management of symptoms primarily with drugs, behavioral therapy, chiropractic manipulation and physical therapy. For presentations with a clear pathological entity, or in cases where conservative treatment is insufficient, surgical intervention may be offered. For carefully selected patient populations, surgical treatment has shown to provide superior outcomes compared to nonoperative treatment for herniation, stenosis, and spondylolisthesis [145] [146] [144]. During surgery, dissection of the vertebral bone and/or disc may be necessary and hardware augmentation may be used to correct deformities, maintain structural integrity of the joint, or to promote fusion of adjacent vertebrae.

1.4. Limitations of Surgical Treatment

As previously noted, a significant portion of back pain is discogenic in nature, and therefore presents without an identifiable pathology. For such cases, Fischgrund observed that:

'The surgical treatment of chronic LBP in the absence of deformity seldom produces clearly beneficial objective results. The surgical treatment of back pain due to disc degeneration (discogenic pain) with spinal fusion provokes considerable controversy.' [37].

In a recent review, the American Pain Society [21] reported that, for *non-radicular* LBP, fusion was 'slightly to moderately more effective than standard (nonintensive) nonsurgical therapy'. For such patients where surgical treatment may be of limited effectiveness, 'Management of LBP should focus mostly on patient education, with short term use of acetaminophen, NSAIDs, or SMT for symptomatic relief of acute LBP, with the judicious addition of opioid analgesics, back exercises, behavioral therapy, or acupuncture for additional symptomatic relief with chronic LBP.' [29]. For this patient group, an alternative treatment option, preferably one that addresses the association between segmental motion and pain is clearly lacking.

A new and novel method to address nonspecific, chronic discogenic LBP by altering the number and type of molecular connections (crosslinks) between collagen fibers that comprise the disc annulus has been proposed[55]. Crosslinks have been used to affect the mechanical behavior of collagen tissue in a wide variety of applications and could be implemented as a means to affect LBP associated with segmental motion.

1.5. Previous Studies of Crosslinking

There are several known chemical agents that will crosslink collagen. Since these agents have different structures and reaction mechanisms, there may be differences in the properties of crosslinked tissue that result from their use. Describing the exact chemical reactions that occur during in vivo crosslinking is difficult as the process depends on the

specific conditions (e.g. pH, temperature, steric effect, and proximity to primary and secondary reactants) at the time of the reaction..

At a basic level, Sundararaghaven [116] demonstrated a dose response relationship between the storage modulus (the elastic component of energy storage in a viscoelastic material) of a hydro gel and the concentration of the crosslinking agent used to treat the gel. For an engineered fibrin based tissue, Bjork [15] showed a clear link between tissue modulus and crosslinking (by controlling UV exposure of the tissue). Moving from synthetic biomaterials to natural tissues, attempts to engineer a set of implant mechanical properties by purposely altering the level of crosslinking has been described for tissues such as the cornea, pericardium, articular cartilage [76], and porcine aortic valve tissue [138]. Recently, Wagner provided a detailed description of how glycation induced crosslinking affects the mechanical behavior of disc annulus tissue, complete with an elegant mathematical model relating the effect of glycation in the constitutive relationship between annular stress and strain [140], [139].

Continuing to move up in scale to the intervertebral joint, Barbir [11] described changes to the axial tension – compression behavior of the rat disc after soaking with a crosslinking agent and Hedman [55] reported differences in the flexion – extension neutral zone behavior of bovine and human discs also after soaking. The neutral zone concept is used to describe the bimodal behavior of the disc. At the 'neutral' position, the disc provides little resistance to rotation. The point where rotation resistance begins to appreciably

increase is the limit of the neutral zone and demarks the neutral zone from the elastic zone. Thus, discs with a small neutral zone will resist rotation sooner than discs with a larger neutral zone. The size of the neutral zone is considered an indicator of intervertebral joint stability, with smaller neutral zones indicating more stable discs [95]. Unlike Barbir and Hedman, Yerramalli [150] investigated an *injection* delivery into the nucleus of the rat disc and did not find a statistically significant effect on the axial tension – compression neutral zone, though they did note a 'trend toward increased stability'.

In addition to achieving the desired change in mechanical behavior, to use crosslinking as a treatment option for LBP will require the effect to be sustainable for a useful time period while in an environment with high potential for enzymatic degradation and while subjected to varying mechanical stress. Currently, reports on the sustainability of post crosslinking effects are scarce, especially with respect to cyclic mechanical loading. Sung [122] demonstrated that crosslinked porcine pericardium had increased degradation resistance compared to fresh tissue as measured by denaturation temperature and free amine content after collagenase digestion and Bigi [14] similarly demonstrated that increased crosslinking of gelatin based films with genipin correlated with decreased release of gelatin. Huang [61] demonstrated that implanted, genipin crosslinked tissue had comparable resistance to in vivo degradation as glutaraldehyde and epoxy fixed controls along with decreased inflammatory reaction. It appears, however, that the only published study directly investigating resistance to mechanical degradation of

crosslinked tissue was by Vyavahare [138] who performed accelerated cyclic mechanical testing of collagen films and porcine aortic valves that were fixed with glutaraldehyde. The films were noted to undergo structural alterations as measured by FTIR after 5 million cycles, and the valve cusps were noted to undergo similar changes after 50 million cycles.

1.6. Purpose

In order to successfully implement a LBP treatment based on crosslinking of the native tissue, it is essential to understand how crosslinking affects the mechanical behavior of the disc tissue. The research presented in the following sections demonstrates some of the basic mechanical properties and behavior of the disc tissue in response to crosslinking. The main goals of the studies were:

- Compare changes in the tensile mechanical response of disc tissue to commonly used crosslinkers under standardized conditions.
- Describe the response of the intervertebral disc in response to a clinically relevant injection of a favorable crosslinker identified earlier.
- Study the changes in degradation resistance of disc tissue in response to combinations of biochemically induced degradation, cyclic mechanical loading induced degradation, and crosslinking.
- Study the changes in delamination resistance of crosslinked disc tissue after crosslinking treatment.

Section 2 describes the optimization of the crosslinker formulations (performed by other contributors) and the subsequent mechanical testing. Using a circumferential tensile test, disc annulus specimens were tested with and without the optimized crosslinking treatments to determine changes to the mechanical properties.

In section 3, Genipin was further explored in a more clinically relevant model by injecting whole motion segments and analyzing the resulting changes in clinical joint stability. The dose response function of the motion segment stability to injection concentration was mapped along with observations of the minimum concentration required to affect a change in stability and the maximum change in stability that could be expected by using a concentration up to 10X the solubility concentration in an aqueous buffer at room temperature.

Effects to the mechanical behavior of gelatin and disc annulus after biochemical degradation, cyclic mechanical compression, and crosslinking treatment with genipin were studied in section 4. Large changes in geometry and compliance consistent with visual observations of induced degradation during cycling were noted in control specimens. Biochemical degradation with trypsin increased the magnitude of the observed changes and genipin crosslinking attenuated the observed changes. Overnight unloaded soaking appeared to recover most of the mechanically induced changes to height and compliance, though visual observations of the tissue suggested that appreciable irreversible degradation had occurred.

Finally, Section 5 used a newly described methodology to evaluate changes in interfacial shear characteristics of the annulus. Crosslinking was seen to increase the yield and peak forces along with resilience of the interlamellar interface.

2. OPTIMIZATION OF PROTEIN CROSSLINKING FORMULATIONS FOR THE TREATMENT OF DEGENERATIVE DISC DISEASE*

2.1. Introduction

Degenerative Disc Disease (DDD) is a debilitating chronic condition [4] with a US economic cost estimated at $100 billion [69]. The spinal disc is an avascular tissue and its cells rely on diffusion and diurnal convection for exchange of nutrients and waste products [133]. During aging, this process becomes gradually impaired [48] as the extracellular matrix of the disc clogs and the adjacent vertebral endplates become sclerotic and calcified. Thus the oxygen content of the disc is reduced and the cells within become progressively more reliant on anaerobic glycolysis as a primary energy source. The resulting lactate production acidifies the tissue [72] and this, coupled to the reduced nutrient influx, results in a decline in the tissue's ability to repair the mechanical damage caused by daily physiological loading and unloading. Over time the tissue's ability to support these loads lessens, leading to fissure formation, stress intensification and loss of disc height. Abnormal bulging of the weakened disc can impinge upon nerve roots, leading to the generation of pain and, in extreme cases, disc herniation.

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* Portions of this work are reprinted with permission from "Optimization of protein crosslinking formulations for the treatment of degenerative disc disease" by Slusarewicz, P., K. Zhu, Kirking B, Toungate J, Hedman T., 2010. Spine **36**(1): E7-13. Copyright 2010 by Wolters Kluwer Health.

The contribution of this dissertation's author is limited to the mechanical tensile and bulge testing, and does not include the chemical optimization of the crosslinking formulations.

Numerous treatment modalities are currently employed for the treatment of DDD [104]. In early stages these include physical therapy and pain management with analgesics and anti-inflammatory agents. While these provide immediate relief, they do not prevent disease progression which is treated with surgical procedures of escalating severity from discectomy, to spinal fusion and artificial disc/nucleus implantation. However, all these therapies are aimed at treating the symptoms of the disease and not to remedying its underlying cause – the degeneration of the disc itself.

Biological approaches aimed at preventing or reversing degeneration have been suggested, including gene, stem-cell and cytokine therapy [2,82,109]. All these approaches, however, are faced with the harsh environment of the pathological disc which is itself not conducive for optimal cellular viability.

It has also been suggested that disc repair and stabilization might be achieved, not biologically, but chemically[22,55]. Such non-surgical exogenous crosslinking therapy (NEXT) offers a promising non-surgical treatment for both retarding the progression and ameliorating the symptoms of DDD. It has been shown that chemical crosslinking of disc tissue leads to an increase in a number of important parameters such as proteoglycan retention, tissue strength, fatigue and tear resistance, joint stability, and a concomitant decrease in disc bulge (and therefore potentially neural compression) under load[23,55,140,150]. Furthermore, crosslinking of collagenous matrices can increase their

permeability[16,26] and, since this can occur within the intervertebral disc[54], crosslinking might reverse the decline in disc cell viability and so facilitate more effective tissue repair.

Numerous chemical crosslinkers have been utilized to modify collagen matrices, such as glutaraldehyde[57], proanthrocyanidins[50], 1-ethyl-3-(3-dimethylaminopropyl)-carbodiim [45], threose [137], genipin(GP) [122] and methylglyoxal (MG) [140]. We have recently characterized the kinetics of these chemical crosslinkers with regard to their ability to crosslink bovine annulus fibrosus tissue [107]. Less understood is how different crosslinkers may affect different changes in mechanical properties of the tissue. Differences in the in mechanical properties after crosslinking treatment may arise from differences in the chemical bond proper [86] or from differences in the reaction mechanics that lead to differences in the resulting crosslink network [66]. We selected GP and MG as reagents for further optimization in this study based on their previous use in the area of biomechanics[16,26,45,50,54,57,118,122,137,140,150] and their relatively low toxicity[98,121].

In order for NEXT to be viable, crosslinkers should be capable of diffusing within the tissue following injection and be active within the environment of the degenerating disc. In this paper, we investigate the diffusion rate of genipin in spinal discs and optimize prototype formulations containing either genipin or methylglyoxal, then compare the mechanical circumferential tensile test properties of bovine annulus after treatment with each formulation.

2.2. Materials and Methods

Genipin was obtained from Challenge Bioproducts Co., Ltd. (Taiwan). All other reagents were from Sigma. Bovine 4-6 month old lumbar intervertebral disc tissue was obtained frozen and thawed at room temperature prior to use. While having similar cross-sectional area as human discs, calf discs have less disc height and are notably non-degenerated. The relative uniformity of these discs leads to minimization of variability in properties.

2.2.1. Diffusion Studies

GP was selected as a representative chemical crosslinker for these studies because its crosslinked adducts turn blue in the presence of oxygen[129,130] 25;26, and can be readily visualized. GP (15mM) was dissolved in phosphate-buffered saline (PBS) and 200µl injected into the discs of individual calf lumbar motion segments, with a 22-gauge needle at a depth of 1cm in the left and right lateral regions, and incubated at 37^{o}C for 1, 3 or 6 hours. The discs were then transected and both halves frozen at -20^{o}C overnight to facilitate the color development of the genipin crosslinked products. Samples were thawed and photographed with a reference scale and the photographs imported into ImageJ software (NIH). Following normalization to the scale, the areas were measured manually by drawing around each zone. In most cases both surfaces of the transected disc were measured, though in a few samples where the cut was too close to the endplate this was not possible. The average of three measurements for each face was used to

determine the area of the diffusion zone in mm^2. Differences between injectates were analyzed using a T-test.

In experiments using surfactants 15mM GP was formulated in either PBS alone or PBS containing 0.1% (w/v) of either SDS or Tween-20. Solutions were injected and analyzed as described above.

2.2.2. Reagent Optimization

GP and MG were selected for further development since they are both relatively small molecules and substantially less toxic than glutaraldehyde [91,98,121], a commonly used crosslinking reagent in the field of tissue engineering.

Approximately 3-5g of frozen bovine annulus was cut into 1-2mm^2 pieces and homogenized in a 50ml Falcon tube in 4^oC distilled water using a homogenizer fitted with a 10mm stainless steel, saw toothed generator probe. The temperature of the suspension did not exceed 25^oC. After 1-2 minutes large particles were allowed to settle and the fine suspension was decanted into a fresh tube. The process was repeated to completion and the tissue harvested by centrifugation at 4500rpm for 5 minutes. Tissue pellets were stored at -20^oC until needed.

Crosslinking extent was assessed by determining the loss in sensitivity of the tissue to digestion by collagenase. Approximately 20-30mg aliquots of homogenized tissue were weighed accurately and 0.5ml of crosslinker added while one sample was treated with

buffer alone. Samples were shaken at 1500rpm and 37^{o}C for 1 hour. Following centrifugation at 10000rpm for 2 minutes, 0.5ml of a 1mg/ml solution of type I collagenase from Clostridium histolyticum in Collagenase Buffer (100mM Tris pH7.5, 10mM CaCl2) was added to the tissue pellets which were incubated as above, but for 20 minutes. Following a second centrifugation, proteolysis was monitored using a colorimetric hydroxyproline assay[9,13,100,102,111] to quantify the release of peptides from the tissue and into the final supernatant. A solution of collagenase alone was used as a spectrophotometer blank and the data were normalized to the results obtained from buffer-treated tissue. Statistical significances were determined using the T-test.

Using this assay, we confirmed previous reports [88,120] that both these crosslinkers are most active at elevated pH [107]. We therefore decided to conduct our experiments under alkaline conditions (pH 9). However, the degenerating human disc is an acidic environment [31,72] and thus is not conducive for the optimal crosslinking activity of these reagents. We therefore buffered our formulations in order to maintain this pH and to counteract the ongoing production of lactate within the degenerating disc.

Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol) buffer has been previously used clinically for the treatment of blood acidosis [67] and therefore represents a potentially useful buffer for this purpose. However, due to concerns that the amine group in Tris might react with both GP and MG and lower the efficiency of crosslinking, we decided to also test a second buffer, EPPS (3-[4-(2-Hydroxyethyl)-1-piperazinyl]

propanesulfonic acid), which lacks amines. We used subsaturating concentrations of both crosslinkers, which we determined using the assays described here (data not shown), in order to discern any positive or negative effects of the buffer on crosslinking. Tissue was crosslinked using 0.1mM GP or 0.4mM MG in 100mM Tris or EPPS buffer at pH 9.

Phosphate ions stimulate the reaction of glucose with proteins, possibly by binding to the target at basic residues adjacent to the crosslinking site and catalyzing the conversion of Schiff base intermediates to stable ketoamine products [131,142]. Since glucose and MG participate in advanced glycation end product crosslink formation via Maillard-type reactions [30,131], we decided to investigate whether phosphate could stimulate crosslinking in our system. Samples were incubated at pH 8 with 1mM GP or 2mM MG at pH 9 or with 0.1mM GP or 0.4mM MG in either 100mM EPPS buffer or 100mM EPPS buffer containing 100mM tri-sodium phosphate.

2.2.3. Mechanical Testing

For circumferential tensile testing, 58 circumferential specimens of annulus were cut from 29 bovine lumbar discs, further cut to narrow a central region. The tensile tests of circumferential annulus specimens were conducted before our observations that Tris exhibited an inhibitory effect on crosslinking and we therefore soaked the tissue at pH 9 in either 10mM GP in 100mM Tris/tri-sodium phosphate or 20mM MG in 100mM EPPS/tri-sodium phosphate at 37°C for 4-hours, or in either buffer alone. Additionally, using kinetic optimizations described previously[107], tissue samples were also prepared

using N-Ethyl-N'-(3-dimethylaminopropyl carbodiimide) (5mM EDC, 100mM MES, pH 6) , Glutaraldehyde (.05% GA by weight, 100mM sodium phosphate, 100mM EPPS, pH 9), and Proanthrocyanidin (.1% by weight, 100mM Tris, pH 8). A description of each cross-linker is given below:

Glutaraldehyde (Figure 1) $C_5H_8O_2$, mw=100.11582g/mol has an aldehyde structure and a reaction rate half life of <5 min at pH of 8-9 [107]. At alkaline pH, GA reacts with amines on the collagen (see figure below) creating a Schiff's base [147]. It can homopolymerize and form complex aldehyde-amine chain copolymers.

Figure 1. Structure of glutaraldehyde and formation of crosslinks in collagen[35].

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Figure 2) $C_8H_{17}N_3$, mw=155.24068g/mol. Reaction occurs between both carboxyl and amine groups on the collagen molecule. The product of the EDC-Carboxyl group then combines with the product of the EDC - amine group forming a 'zero-length' crosslink and urea excrete-able products[92]. EDC cannot homopolymerize, but could contribute to formation of

amine-carboxyl based chains [127] to form complex co-polymers. EDC has a reaction rate half life of <5 min at pH 6 [107].

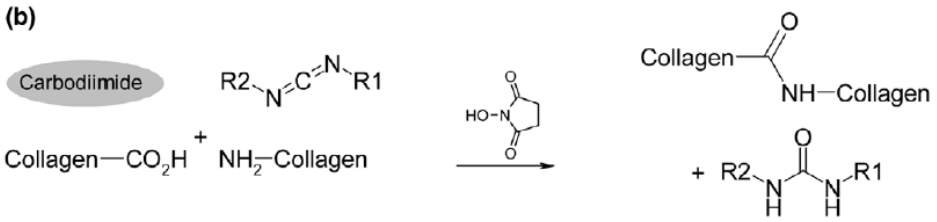

Figure 2. Structure of EDC and formation of crosslinks in collagen[35]

Proanthocyanidin (Figure 3) $C_{31}H_{28}O_{12}$, mw=592.54682 g/mol has a reaction half life of < 5 min at pH 5-9[107]. Reaction of proanthocyanidin with collagen is thought to result in stable hydrogen boding with the collagen amines[50].

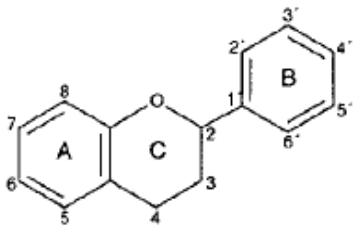

Figure 3. Structure of Proanthocyanidin[50].

Methylglyoxal (Figure 4) $C_3H_4O_2$, mw= 72.06266 g/mol has an aldeyde structure and at pH 8-9, the reaction half-life is 14-20 minutes[107].

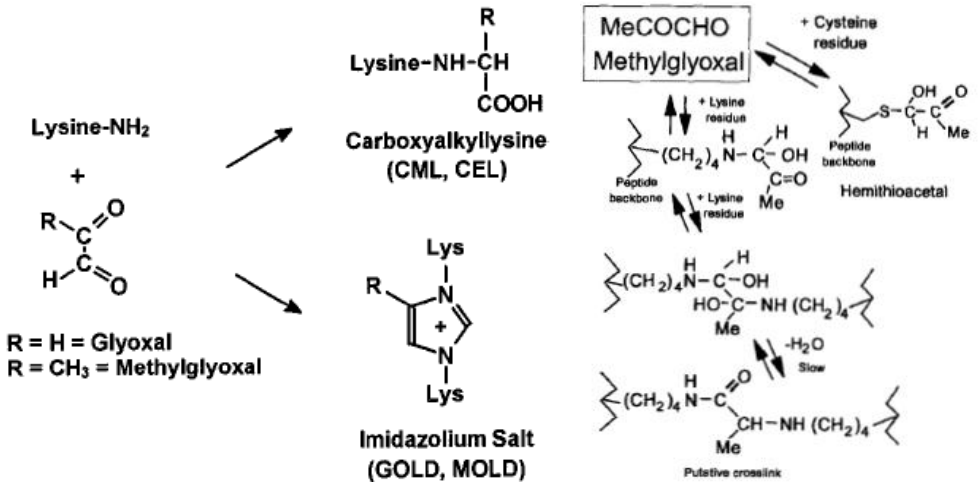

Figure 4. Structure of methylglyoxal[40] (left) and proposed crosslink formation in collagen(right)[80].

Genipin (Figure 5) $C_{11}H_{14}O_5$, mw=226.22586 g/mol has a reaction half life of 10-18 minutes at pH 8-9[107]. Reaction begins with nucleophilic attack on the C3 carbon followed by opening of the dihydropyran ring. Once opened, an aldehyde structure is formed that will react with amine groups (Figure 4) on the collagen. The genipin dimer can form both homopolymers as well as copolymers with amines [129].

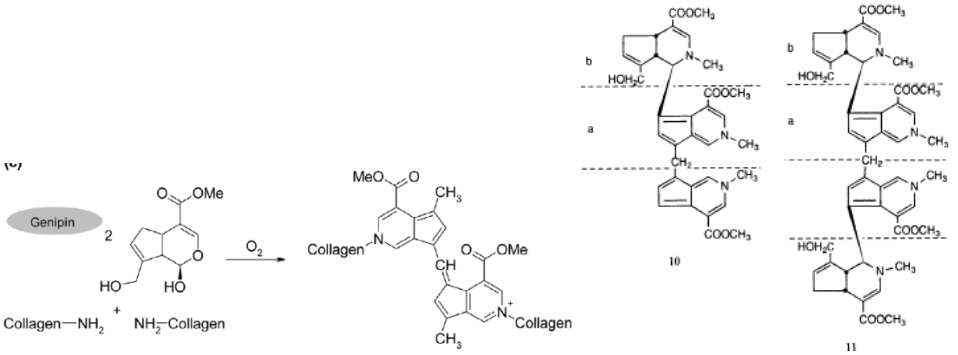

Figure 5. Structure of genipin and crosslink formation in collagen (left,)[35] and complex polymer formation (right)[129].

The smallest cross-section in the necked-down region of each sample was measured using a rotating laser micrometer. The specimen was positioned in custom clamps and a tensile test was run at a constant displacement rate of 0.3mm/sec. Peak modulus, ultimate tensile strength, yield stress (using a 0.1% offset method), yield strain, and resilience (total energy absorbed at the yield point) were calculated from the stress-strain data. Data were expressed as the percentage change in the parameter in a treated specimen compared to the mean of the untreated samples. A Mann-Whitney non-parametric test was used to determine significance of mean differences between groups ($\alpha \leq 0.05$).

Disc bulge measurements were conducted on twelve calf lumbar motion segments that were potted in polyurethane and randomly assigned to receive either an injection of 10mM Genipin in 100mM EPPS/100mM tri-sodium phosphate, pH 9 or an injection of buffer alone. Discs were injected with 0.75ml of solution at both antero-lateral positions

(i.e. 1.5ml per disc) using a 1ml syringe and a 22 gauge needle, and then incubated for 4-hours at 37°C under an 89N axial load. After incubation, specimens were clamped into place on a materials test system (TestResources 100R) such that the load axis was parallel to the specimen's anatomical superior-inferior axis and centered on the disc's center of mass. Cyclic compressive load from 0N to 400N was applied at 0.05 mm/sec for 40 cycles to condition the specimen. A rotating LK-081 laser micrometer (Keyence) was used to measure the anterior surface of the disc at three heights (midline and 2mm above and below) at static loads of 50 and 400N. Disc surface position was analyzed for each applied load and at each height using custom Octave code. Bulge was calculated as the difference in disc surface position at each height between the 50 and 400N loads. One specimen from the treatment group was 2 standard deviations away from the mean of the remaining samples and was discarded as an outlier. Statistical significance was determined using the Mann-Whitney-test.

2.3. Results

The efficacy of NEXT will depend on a number of factors, including the ability to deliver the crosslinker over a large portion of the annulus and the efficiency of the crosslinker once introduced into the milieu of the degenerating disc. In the case of delivery, we first assessed the diffusion of GP, a possible therapeutic crosslinker, following injection into the annulus of bovine spinal discs.

GP diffusion following injection formed clear "zones" (Figure 6) whose area increased time-dependently (Figure 7). We also examined whether two surfactants (SDS and Tween-20) could enhance diffusion of these markers (Figure 8), but only SDS did so significantly (by approximately 30%, $p=0.021$). We therefore decided to not pursue surfactants, since the effect we observed with SDS was modest and did not justify the added complications of its inclusion in a formulation.

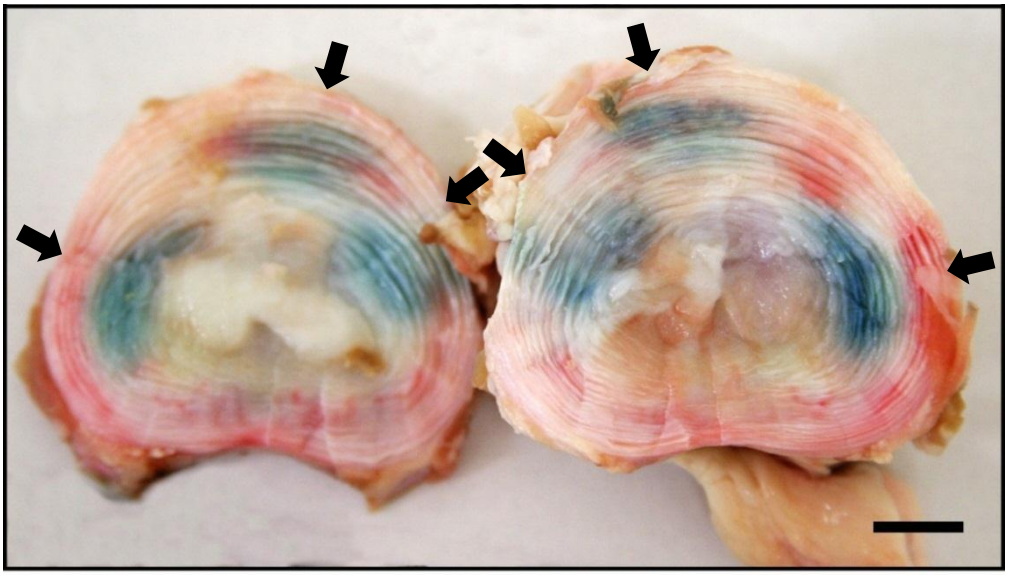

Figure 6. Example of genipin diffusion in a calf lumbar spinal disc. GP was injected into the disc of a single motion segment and incubated for 1 hour at 37°C. Arrows indicate the injection points. Bars = 10mm.

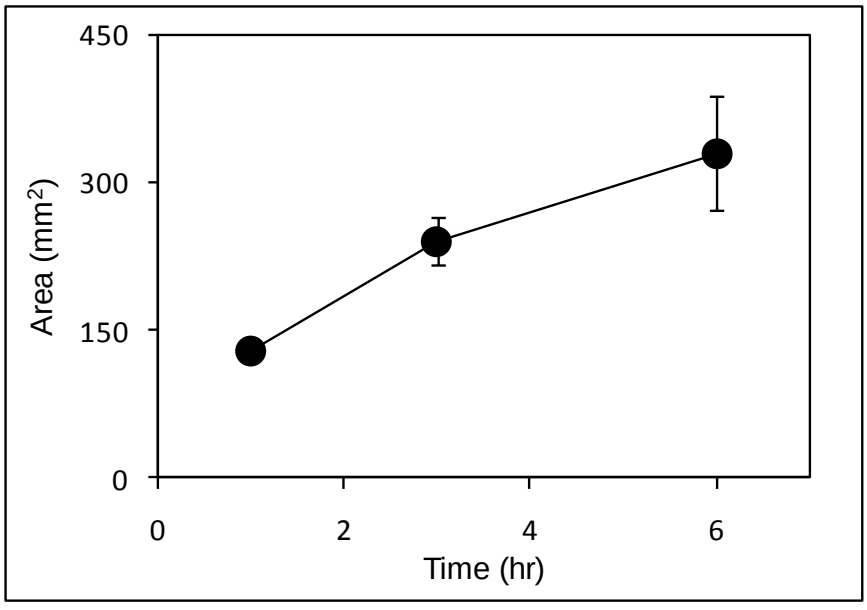

Figure 7. Diffusion kinetics of genipin. GP was injected into bovine lumbar discs and incubated for 1 (n=7), 3 (n=4) or 6 hours (n=4) at 37°C. Data are presented ± SD.

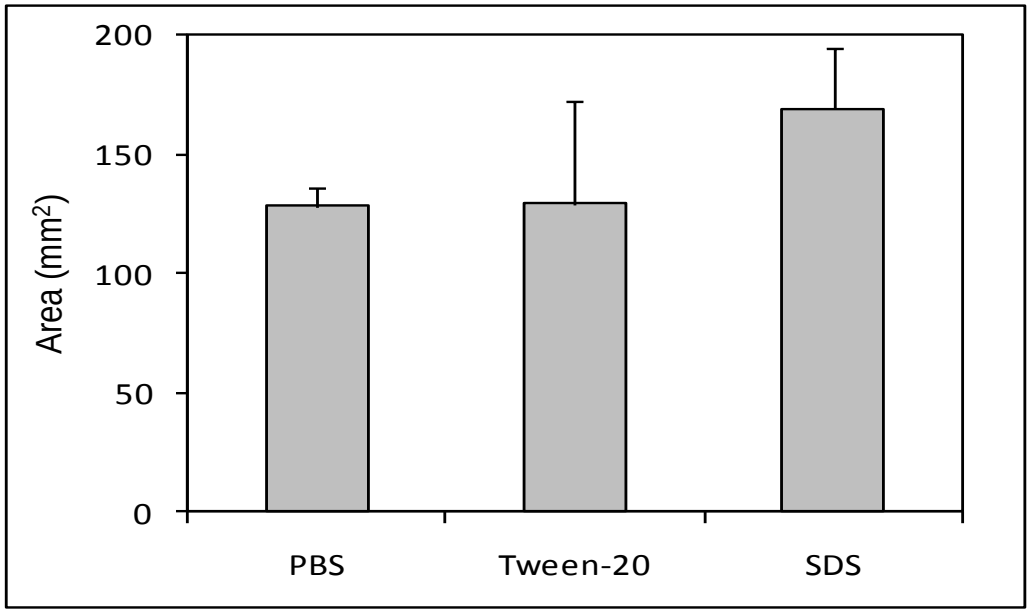

Figure 8. Effect of surfactants on diffusion of genipin. GP in PBS (n=7) or in PBS containing Tween-20 (n=3) or SDS (n=5) was injected and incubated for 1-hour. Data are presented ± SD.

When testing the effects of buffers on crosslinking we found that it was less efficient in the presence of Tris buffer when compared to EPPS (Figure 9) for both GP ($p=0.0013$) and MG ($p=0.0021$). Under these conditions GP crosslinking was 47% more efficient in EPPS while the enhancement for MG was 44%. In addition, phosphate ions increased crosslinking by GP by 72% at pH 8 ($p<0.001$) and by 44% at pH 9 ($p<0.01$), while MG was 50% more efficient at pH 8 ($p<0.001$) but unaffected at pH 9 (Figure 10). Such effects were not due to the added sodium in the mixture since tri-potassium phosphate caused similar effects (data not shown).

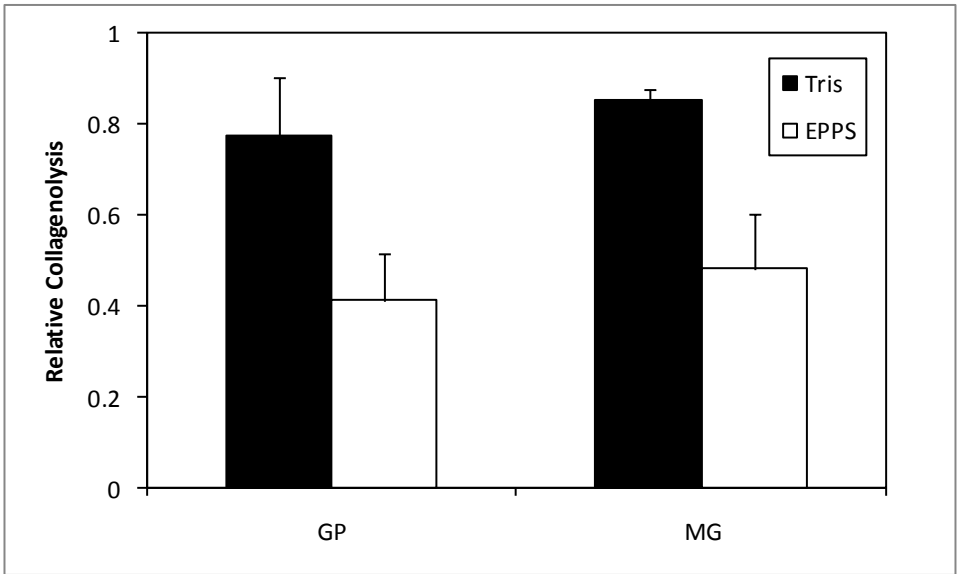

Figure 9. Buffers effects on genipin and methylglyoxal crosslinking. Tissue was incubated with GP or MG at 37°C for 1 hour in either Tris or EPPS buffers at pH 9. Crosslinking was quantified and normalized to untreated tissue. Data are presented ± SD (n=5).

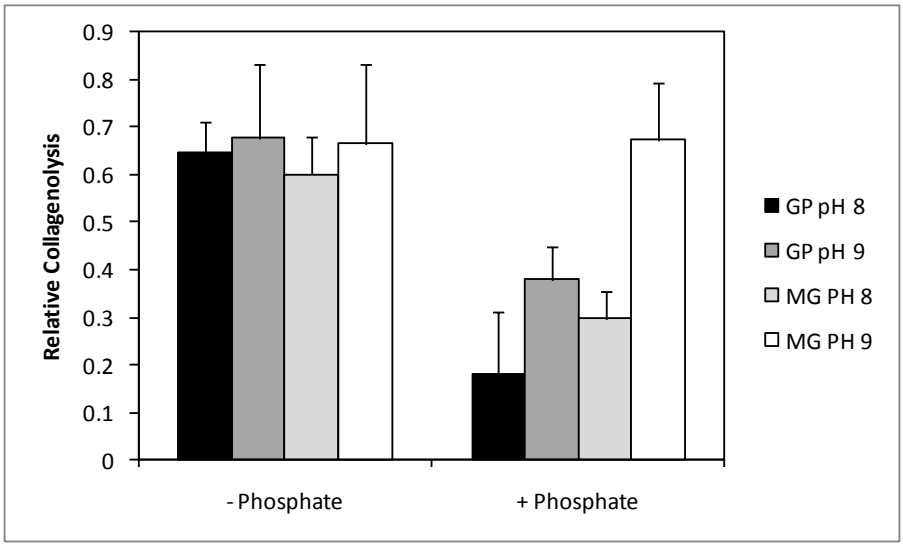

Figure 10. Effect of phosphate on genipin and methylglyoxal mediated crosslinking. Tissue was incubated with GP or MG at pH 8 or 9 at 37°C for 1 hour, in the presence or absence of phosphate. Crosslinking was quantified and normalized to untreated tissue. Data are presented ± SD (n=5).

The mechanical properties of tissues treated with crosslinker were compared to those treated with buffer alone using a circumferential tensile test. GP elicited increases of 66%, 36%, and 57% in yield stress, yield strain, and resilience or energy required to generate non recoverable deformation (P= 0.012, 0.048, and 0.019, respectively), to the annulus tissue. Peak modulus trended 23% higher with GP treatment, but this difference was not statistically significant. In contrast, MG treatment resulted in a 69% increase in modulus (P = 0.009), and trended higher in yield strain (46%) and ultimate tensile strength (27%), although these increases were not statistically significant. A summary of all our circumferential testing data is shown in Figure 11 as a percent change from each treatment's respective control. Additionally, in Table 1, the mean values of the circumferential tensile test properties are shown for GP and MG and their respective control group.

Of all the crosslinkers tested, EDC showed the largest magnitude of change for all values except peak modulus. EDC treated tissue yield stress was 322 % greater than controls, yield strain was 245 % greater*, resilience was 327 % greater, peak modulus was 131% greater, and UTS was 169% greater.

GA treated tissue values were less than controls for all values except peak modulus. Yield stress was 64 % less, yield strain was 89 % less, resilience was 64 % less, and UTS was 79% less. Only peak modulus was greater (160%) after treatment with GA,

though none of the measured changes in GA were statistically significantly different from controls.

PA showed the smallest overall magnitude in change in all values except UTS. PA treated tissue yield stress was 111 % greater than controls. Yield strain was 107 % greater, resilience was 111 % greater, peak modulus was 93 % less, and UTS was 120 % greater. None of the measured changes in PA were statistically significantly different than controls.

We supplemented these data by measuring the effect of GP in optimized buffer with respect to its ability to reduce disc bulge under load following injection (a therapeutically more meaningful method of delivery). Specimens treated with buffer demonstrated a mean disc bulge of 0.28mm (±0.07) while GP reduced the value to 0.17mm (±0.07). This 37.5% reduction was statistically significant ($p=0.04$).

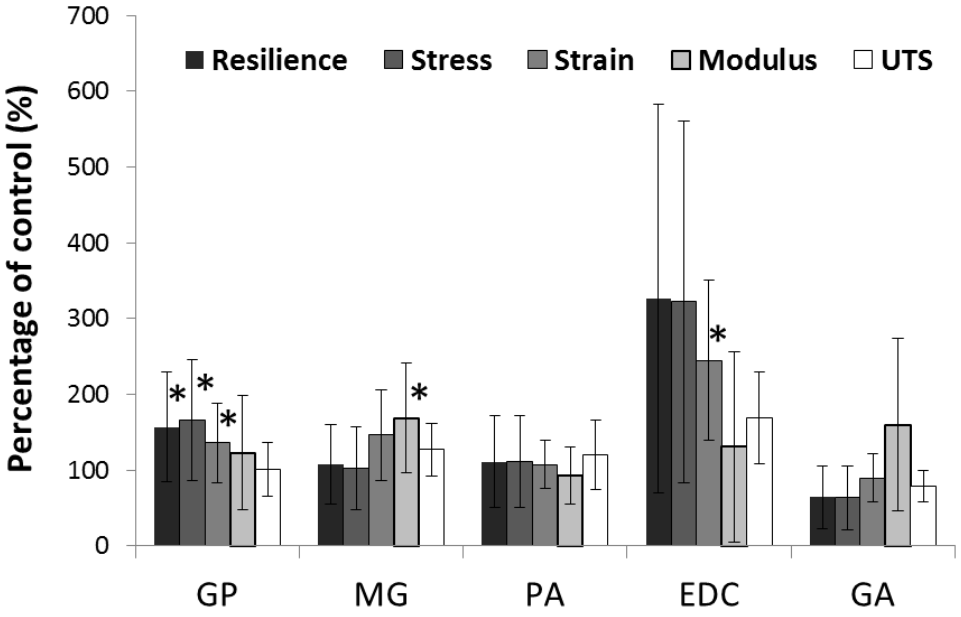

Figure 11. Circumferential tensile mechanical properties of crosslinked annulus tissue. Tissue was crosslinked with genipin (GP), glutaraldehyde (GA), proanthrocyanidins (PA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiim (EDC), or methylglyoxal (MG). Data are presented as mean percentage change relative to sham ± SD. * Indicates statistical significance relative to sham treatement, $p<0.05$.

Table 1. Summary of circumferential tensile testing data. Annulus specimens were treated with either GP or MG, or sham-treated with the respective buffer, and then subjected to tensile testing. Five parameters were measured and the results are reported as the mean and standard deviation of each group. Units for the parameters are MPa (resilience, yield stress, UTS – ultimate tensile strength), mm/mm (yield strain) and GPa (peak modulus). Treated results marked with an asterisk denote a significant difference relative to the corresponding sham.

	Resilience	Yield Stress	Yield Strain	Peak Modulus	UTS
GP Sham	1.09±0.59	0.96±0.59	5.08±2.37	14.32±5.58	8.08±2.56
GP Treatment	1.71±0.80*	1.59±0.76*	6.90±2.64*	19.15±10.74	8.81±2.87
MG Sham	1.83±1.50	1.72±1.49	5.08±2.61	9.40±3.10	6.61±3.07
MG Treatment	1.97±0.97	1.76±0.94	7.42±3.03	16.40±6.81*	8.49±2.29

2.4. Discussion

NEXT holds the promise of an effective and nonsurgical treatment for DDD and would be accomplished by the injection of chemical crosslinking agents into the affected spinal disc. NEXT would act to stabilize and strengthen the disc, and promote maintenance of hydration and the diffusion of nutrients and oxygen into, and waste products out of, the tissue.

We have shown GP is capable of substantial diffusion following injection into a disc (Figure 7) and that SDS, but not Tween-20 can enhance its diffusion (Figure 8). SDS is a harsh anionic surfactant and its ability to denature proteins may have "loosened" the annulus matrix more than the milder, non-ionic Tween-20, thus leading to faster GP diffusion. The harshness of SDS coupled to its relatively modest effect led us to exclude it from further consideration.

Formulation optimization showed that GP and methylglyoxal (MG) crosslinking was less efficient in Tris than in EPPS buffer (Figure 9), suggesting that the use of amines should be avoided. We also demonstrated that phosphate ions can enhance the reaction of both GP and MG (Figure 10). The inclusion of phosphate into the formulation can also broaden the buffering capacity of the solution (data not shown), further counteracting the acidity of the degenerating disc. The effect of phosphate appeared to be pH dependent. Since both GP and MG activities increase with pH this may have been due to the elevated activity masking the phosphate effect. MG activity is more sensitive

to pH than GP [107], and was also unaffected by phosphate at pH 9. MG's reactivity at this pH may have been so high that it could not be further enhanced by phosphate. Nevertheless, we decided to retain phosphate in the MG formulation, since we would expect the pH to drop following injection into the acidic environment of the disc.

The enhancement of GP-mediated crosslinking by phosphate was surprising and may be due to the involvement of aldehyde groups in the GP crosslinking reaction. It has been postulated that the initial step in the reaction involves a nucleophilic attack on the C-3 olefinic carbon atom in GP by an amine, resulting in the opening of its dihydropyran ring, followed by a second attack of the attached amine on the resulting aldehyde group25. This second attack closes the ring with the substitution of the amine nitrogen (and attached moieties) for the original oxygen in the GP structure. Crosslinking then occurs through polymerization of the GP derivatives and the molecules to which they are coupled [129,130]. Alternatively, the hemiacetal within the dihydropyran ring could undergo consecutive steps of hydration, opening the ring to produce two aldehyde groups40 which could either react with different protein-bound amines to form a crosslink directly or react with a single amine and then follow the reaction described above. In either pathway Schiff bases form via reaction of amines with aldehydes and their rearrangements could be catalyzed by phosphate ions in a manner similar to those formed during protein glycation.

Using a circumferential tensile test, we examined the effect of crosslinking on annulus tissue using optimized buffer formulations. As anticipated, different crosslinking agents were shown to produce significant differences in how they changed the tensile properties. However, this study cannot explain why the different crosslinkers altered tensile mechanical properties in different ways. The difference in the chemical bond has been suggested as an explanation for differences in the alteration of mechanical properties after crosslinking bovine pericardium. While GA, GP, and MG have all been shown to act primarily on amine structures, EDC has been shown to react with both amine and carboxyl structures. Caroboxyl and amine groups are at different locations on the collagen molecule and that difference geometrical location may influence the mechanical behavior exerted by the crosslink.

Others have suggested that the dynamics of the network formation influences the changes in mechanical properties [38]. In this study, all the formulations used were based on data to optimize reaction rate in homogenized tissue while the treatment of the mechanical test specimens was performed by soaking in solutions with an excess of crosslinker. Zhu[152] described the extent of crosslinking in soaked bovine annulus specimens using ratios of transitional enthalpies measured from differential scanning calorimetry (DSC). There is a trend between the extent of crosslinking as measured by the ratios Zhu reported and the magnitude of changes in mechanical properties observed in this study, suggesting that the changes in mechanical properties were dependent on the extent of crosslinking achieved by the optimized formulations. Thus, the very large

changes in mechanical properties observed with EDC were due to the ability of the EDC formulation to penetrate into the tissue and form a large number of crosslinks. Conversely, the lack of effect from GA was due to an inability of GA to penetrate into the tissue to form large numbers of crosslinks creating a highly treated zone surrounding a center of minimal treatment. While GA is a relatively small molecule (MW ~ 100) which would imply greater access to potential crosslink sites, its high reactivity likely hampers crosslink formation deeper in the tissue.

We also tested GP in a more clinically relevant manner by injecting the crosslinker directly into bovine lumbar discs using an EPPS/phosphate buffer. The GP treatment significantly reduced the bulge of the treated discs under a 400N axial load by 37.5% compared to discs injected with buffer alone and demonstrated the potential of NEXT when applied by injection. The relevance towards decompression of adjacent neural structures (radiculopathy) and nociceptor excitation thresholds (discogenic pain) due to this degree of disc bulge reduction should be further explored in future studies using human tissues. Increases in disc bulge has been associated with increasing disc degeneration in the clinic [20]. It is expected that increased levels of degeneration combined with an increased disc height should amplify both the amount of disc bulge and the NEXT reduction of bulge in human discs.

In order for NEXT to be therapeutically viable, it needs to exhibit an acceptable biocompatibility profile. Two areas of concern could be the effect of the formulation on

the viability of the cells of the disc and the possibility of neurotoxicity from leakage of crosslinkers from the disc and into the spinal canal or onto nerve roots. We are in the process of conducting studies to address these issues, but our preliminary data indicate that both formulations do not affect cell viability when injected subcutaneously (data not shown).

Having developed suitable, optimized formulations, we next plan to determine their effects on a broad range of mechanical parameters of human spinal motion segments following injection, as a prelude to clinical testing of NEXT technology in the clinical setting.

2.5. Conclusion

Formulations lacking amines and containing phosphate ions appear to be promising candidates for clinical use of the crosslinkers genipin and methylglyoxal. Treatments of bovine annulus fibrosus with commonly used crosslinker reactants formulated for optimal reaction rates produced changes in tensile mechanical properties that were unique to each reactant. EDC produced the overall largest changes, while genipin produced more uniform changes to the parameters measured.

3. THE DOSE RESPONSE RELATIONSHIP BETWEEN INTERVERTEBRAL DISC FLEXION-EXTENSION NEUTRAL ZONE METRICS AND INJECTED GENIPIN CONCENTRATION

3.1. Introduction

The intervertebral disc is a composite structure generalized as consisting of three major components: the nucleus, annulus fibrosus, and endplates. In healthy discs, the nucleus is at the center of the disc and is a gel-like matrix of water, collagen, proteoglycans, and other proteins that pressurizes in response to compressive loading. The annulus fibrosus surrounds the nucleus, is composed of alternating layers of obliquely aligned collagen fibers and restrains the pressurized nucleus. The endplates anchor the superior and inferior borders of the disc to the adjacent vertebral bodies and allow nutrients to pass into the annulus and nucleus [4,18,28,59].

Low back pain (LBP) affects an estimated 60-80% of the general population at some point [71] creating a substantial economic burden on Western society [33]. Degeneration of the disc is often associated with LBP, but is also common in pain-free individuals [58,94]. With damage or degradation, the normal load bearing capabilities of the disc are altered and may result in abnormal loading and motion of the intervertebral joint. Common surgical treatments for painful, degenerated and damaged discs include discectomy, fusion, or a combination of discectomy and fusion. Both discectomy and fusion are limited in their ability to restore motion and stability of the intervertebral joint, thus great

efforts have been expended developing numerous partial and total disc replacements, some with purported regenerative potential. However, consistent long-term clinical results have not been demonstrated[10,43,106].

Because of the limitations of discectomy, fusion, and disc replacements, these approaches are most often applied in cases where the disc is severely damaged or degraded. Alternatively, therapies seeking to treat the native tissue in early stages of degradation and damage may be an effective means to slow or halt further degeneration. For example, when damage is mostly contained within the annulus, exogenous crosslinking of the damaged annulus tissue may be able to restore the mechanical function and load carrying abilities of disc tissue [55,108,150]. Further, recent in situ studies have demonstrated that delivery of a crosslinking agent could be accomplished using a fluoroscopy guided percutaneous injection into the annulus fibrosus at the affected level [123], similar to existing techniques developed for vertebral biopsy, discography, and chemonucleolysis of herniated nucleus material present in the annulus [49,73,77]. If the disc has severe degradation of the nucleus, but mild to moderate degradation of the annulus, an artificial nucleus replacement device in combination with crosslinking of the native annulus may be sufficient to restore vertebral-disc joint function [10,42].

Crosslinking collagenous soft tissues to alter their mechanical characteristics is an increasingly common practice and has been described for porcine aortic valve xenoplants [27,119],the cornea[5,110], and also for hydrogels[14,116]. It follows that if native soft

tissues contribute to the function of a joint, then crosslinking those tissues will result in changes to joint function. However, the effect of such tissue alterations on joint function may not be obvious, nor is it obvious how much crosslinker is required to affect meaningful changes. Adding further complexity, different crosslinkers demonstrate different mechanical effects on the same tissue [108] which may extend to different effects on the associated joint.

For the intervertebral disc, the neutral zone is an important parameter in the characterization of segmental stability for the purpose of treating low back pain (LBP). In a clinical setting, the ability to alter the intrinsic stability of the intervertebral joint using an injected crosslinking agent would be an invaluable tool in the treatment of LBP. Two previous in-vitro studies of the disc demonstrated that crosslinking the disc by soaking in genipin produced changes in joint behavior: Hedman et al [55] reported a 41% decrease in flexion - extension neutral zone length in bovine lumbar motion segments while Barbir et al [11] found a 965% increase in axial neutral zone stiffness and a 58% decrease in axial neutral zone length for rat motion segments. Another study of the intervertebral disc which injected the nucleus pulposus with genipin did not find a significant difference in axial neutral zone length or stiffness, but did note that both parameters trended toward increased stability [150].

Therefore while crosslinking the intervertebral disc by soaking in genipin has shown the ability to affect neutral zone motion, the ability to affect the flexion – extension neutral

zone with an injection mode of delivery has not been established, nor has the relationship between neutral zone changes and concentration of the injected genipin been demonstrated.

This relationship between crosslinker concentration and mechanical effect has been characterized for gel films and the cornea [5,14,116]. Therefore, the objective of this study was to map the changes in the metrics of the flexion-extension neutral zone of the intervertebral disc with injections of increasing genipin concentration.

3.2. Materials Methods

3.2.1. Specimen Preparation

Twenty fresh frozen bovine tail specimens were obtained from a local supplier and kept frozen until ready to use. All specimens were prepared by stripping the tissue external to the disc and vertebrae, cutting through adjacent vertebrae and potting the hemi-vertebrae in cylindrical molds with fast setting, mildly exothermic, rigid urethane. During preparation, potting, and subsequent testing, specimens were closely monitored, wrapped with moist gauze, and sprayed with a saline mist to protect against dehydration.

To equalize initial hydration for the specimens, all specimens were given bilateral 0.5mL annular injections of phosphate buffered saline (PBS) and then wrapped in PBS-moistened towels and placed under a 0.12 MPa axial load overnight at 4 °C. Preliminary studies demonstrated that this protocol of fluid injection followed by several hours of

low force compression does not substantially affect the joint's neutral zone length and slope during simulated flexion-extension.

3.2.2. Treatments

Following overnight loading, specimens were tested in flexion-extension to provide a baseline and then randomly administered an injection treatment with buffered (50mM EPPS Phosphate, pH9) genipin solution at 0, 20, 40, 80 or 400 mM using bilateral 0.5 mL annular injections. Each treatment group had four specimens. The 80mM and 400 mM genipin concentrations were greater than the solubility limit for the genipin; therefore DMSO was used as a cosolvent to prevent precipitation of the genipin out of solution. Immediately after injection, specimens were wrapped with PBS-moistened towels and incubated under a 0.12 MPa axial load for 4 hours at 37 °C followed by overnight storage under load at 4 °C. It should be noted that the specimens were intact, healthy discs and this model was not intended to simulate a clinically unstable disc.

3.2.3. Mechanical Testing

Prior to testing, specimens were removed from the refrigerator and allowed to equilibrate to room temperature for at least 1 hour. Specimens were then placed in the testing system (Test Resources 100R1000, Minneapolis, MN) by clamping to the inferior pot with the load axis of the system aligned along the anterior – posterior axis of the specimen as diagrammed in Figure 12.

A more typical loading method described in similar testing is the direct application of a bending moment which may be also accompanied by a constant compressive force.

Advantages of a directly applied moment include having a constant moment acting across multiple motion segments and isolation of the mechanical behavior to loading components. As the objective of this study was to investigate if injection delivery of the crosslinking agent could alter the mechanical behavior relative to a sham treatment under identical loading conditions, the traditional bending moment loading method was not critical; therefore, the simpler to implement shear-compression-flexion moment methodology was used.

Specimens were cycled for 19 cycles to minimize early transient differences between cycles. The loading portion of the 20th cycle was used to analyze the segment's flexion-extension neutral zone. Neutral zone length and stiffness were calculated from a first order fit to the moment – angle data, similar to that described by Hedman et al and Yerramalli et al [55,150]. For this analysis, the neutral zone region was defined as the angular displacements for which the residual of the linear fit was less than 10% of the measured moment. A graphical description of the neutral zone and the parameters used to characterize the neutral zone is given in Figure 13. In addition to neutral zone characteristics, range of motion (ROM) and the instability score (ISS) (ISS=the neutral zone length divided by the product of ROM and neutral zone stiffness[55]) were also calculated.

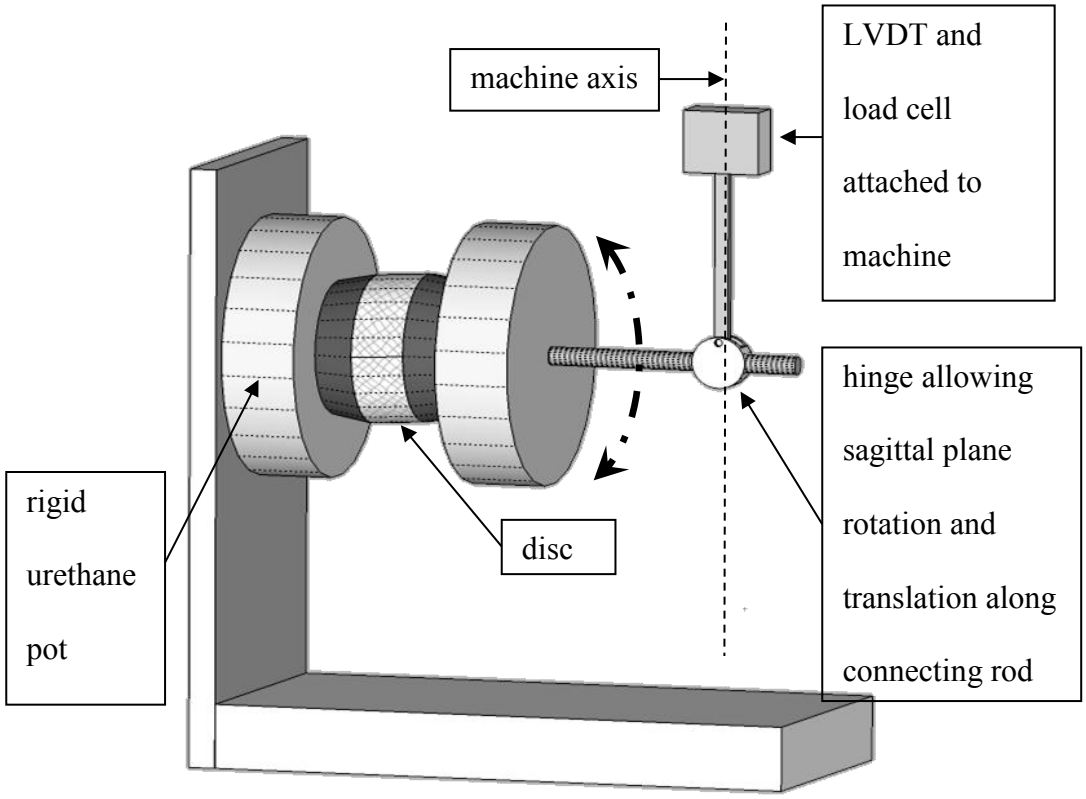

Figure 12. Schematic of the experimental setup used to simulate and measure intervertebral disc stability. The machine applies translation to the specimen through a sliding hinge and connecting rod producing flexion – extension motion of the motion segment (dash-dot line).

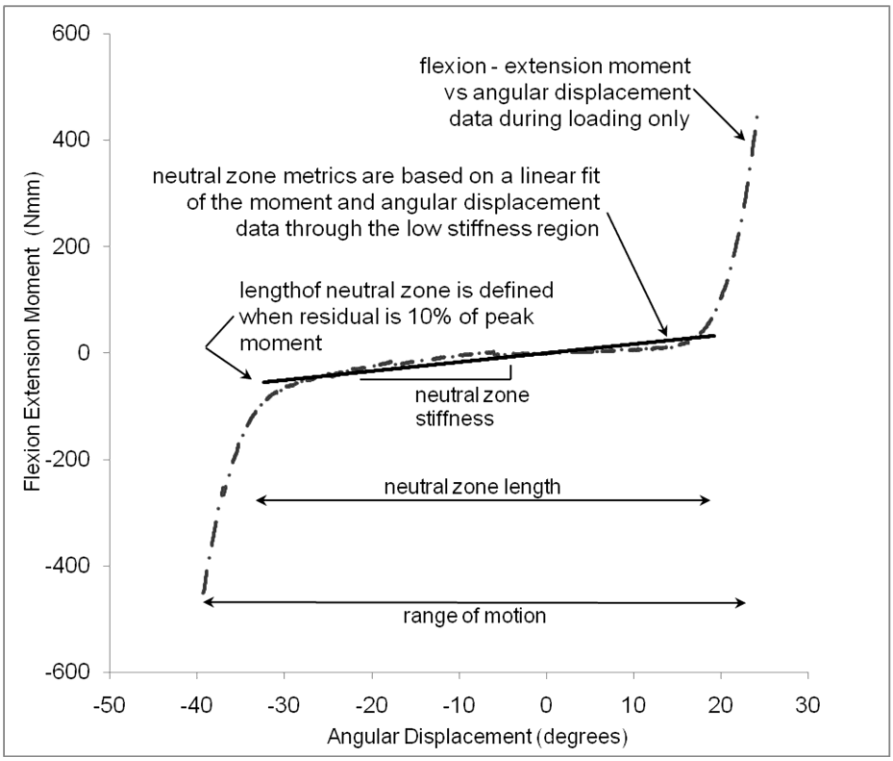

Figure 13. Graphical description of neutral zone characteristics and range of motion. The dash-dot line is the collected force and linear displacement data transformed into moment and angular displacement about the disc center. The solid line is a linear fit through the data in the low stiffness region. The end of the neutral zone is defined as the point where the residual between the fit and data reaches 10% of the peak applied moment. The slope of the fit is the neutral zone stiffness and the total angular displacement of the fit is the neutral zone length. Range of motion is defined as the total angular displacement between maximum and minimum applied moment.

3.2.4. Statistical Analysis

Statistical significance of genipin injection concentration on pre-post treatment changes in range of motion, neutral zone length, neutral zone stiffness, and Instability Score at each treatment level versus the sham treatment was tested using the Kruskal Wallis nonparametric test.

3.3. Results

The effect of genipin treatment on the flexion – extension neutral zone parameters was dependant on the concentration of genipin used in the treatment. The study results are detailed in Table 2. The parameters that produce the instability score (range of motion, neutral zone length, and neutral zone stiffness) and the instability score were all significantly affected by at least one genipin treatment level, $p<0.005$.

Compared to the sham injection, changes in ROM and neutral zone length demonstrated significant decreases for the 80 mM and 400 mM treatment groups. ROM decreased by 5.7% with the 80mM injection and by 21.8% with the 400mM injection. Neutral zone length decreased by 9.1% with the 80mM injection and by 26.3% with the 400mM injection.

Neutral zone stiffness demonstrated significant increases for the 40 mM and greater concentrations compared to the sham injection. At 40mM, neutral zone stiffness increased by 48.1% and at 80mM stiffness increased by 49.9%. With the 400mM injection, neutral zone stiffness increased by 242% which was the largest change of all investigated variables at any treatment level. Unexpectedly, neutral zone stiffness *decreased* with the 20mM treatment by 9.7%. Based on the range of values and the trends observed in all the neutral zone parameters, the observed decrease in neutral zone stiffness at 20mM may indicate the amount of error in the measurements, approximately 10%, rather than a destabilizing effect of genipin.

Table 2. The mean range of motion (ROM, degrees), neutral zone Length (NZ Length, degrees), neutral zone stiffness (NZ Stiffness, N/degree), and instability scores (ISS, N^{-1}) of the bovine motion segments pre and post treatment. Percent difference in each metric was calculated relative to the pretreatment value. Statistically significant differences ($p<0.05$) are shown in bold.

		Genipin Concentration (mM)				
	Metric	0	20	40	80	400
Pre Trt	ROM	43.6	32.0	43.3	34.8	46.0
Post Trt	ROM	46.7	34.9	45.0	32.7	35.8
% difference	ROM	7.3	9.1	4.2	**-5.7**	**-21.8**
Pre Trt	NZ Length	34.6	25.0	35.4	27.2	37.4
Post Trt	NZ Length	37.8	27.7	37.0	24.5	27.4
% difference	NZ Length	9.1	11.4	4.7	**-9.1**	**-26.3**
Pre Trt	NZ Stiffness	3.1	4.7	3.0	4.3	2.9
Post Trt	NZ Stiffness	3.2	4.2	4.3	6.1	9.5
% difference	NZ Stiffness	0.8	-9.7	**48.1**	**49.9**	**242**
Pre Trt	ISS	0.255	0.942	0.305	0.191	0.302
Post Trt	ISS	0.257	0.944	0.205	0.124	0.082
% difference	ISS	0.99	0.18	**-30.0**	**-31.3**	**-72.0**

In a similar manner to neutral zone stiffness, instability score did not significantly change relative to the sham with the 20mM injection, but did significantly change for injection concentrations of 40mM and greater. At 40mM, ISS decreased by 30.0% of pretreatment and at 80mM ISS decreased by 31.3%. With the 400mM injection, ISS decreased by 72% of the pretreatment valued.

3.4. Discussion

Our study data demonstrate that annular injections to the disc with varying concentrations of genipin altered the flexion – extension neutral zone of the disc as measured by neutral zone stiffness, neutral zone length, ROM, and instability score. While the discs themselves were healthy and presumably clinically stable before treatments, the overall effect of the genipin injections on the neutral zone metrics was such that neutral zone stability of the disc increased with increasing genipin concentration. However, the changes in the neutral zone metrics and ROM were not uniform across treatment concentrations.

For the studied treatment amounts, none of the measured parameters demonstrated significant changes toward increased stability for injection treatments of 20 mM or less. For the 40mM treatment, the effect of the crosslinks is limited to the neutral zone stiffness and instability score. With the 80mM treatment neutral zone length and ROM were also noticeably affected, and while stiffness was significantly different than the sham treatment, it wasn't appreciably changed from the 40mM treatment. Above 80mM, all metrics continued to be affected by even greater amounts.

At every effective concentration, the affect to neutral zone stiffness was relatively greater than the affect to neutral zone length or ROM. This result agrees with an earlier hypothesis of Hedman et al [55], suggesting that crosslinking may reduce slack in the fibers so that they are more fully engaged in the low stiffness region of the motion curve:

"This increase in fiber tautness could be from a combination of intrinsic planar waviness (crimp) reduction, and/or reduction of fatigue or creep incurred non recoverable deformations." An earlier engagement of a larger percentage of fibers would have minimal effect outside of the neutral zone range of motion. However this study cannot determine whether the greater relative effect on neutral zone stiffness compared to neutral zone length or ROM results from interfibril, intrafibril crosslinks, or if the difference is due to secondary effects such as changes in water content and flow through the more highly crosslinked structure.

Yerramalli et al [150] reported that 100µL of 1% genipin treatment in the nucleus pulposus of rat motion segments "tended to increase stability" when tested in axial tension-compression, but did not find a significant difference. Based on the reported size of the rat disc (approximately 10mL), their 100µL of 1% genipin resulted in a genipin density of approximately 0.01 mg / ml of disc. In comparison, bovine tail discs are on the order of 300mL, the necessary injection concentration needed to achieve the same genipin density in a bovine tail disc with a 1mL injection is approximately 10mM. Using the 0mM and 20mM to estimate the effect of a 10mM concentration, it appears that there would not be a meaningful change toward increased stability. Therefore, the 0.01 mg/ml genipin density does not appear to be sufficient to alter neutral zone metrics toward increased stability.

In contrast with an injection, soaking in an excess supply of genipin should allow large amounts of genipin to penetrate into the disc tissue, providing sufficient soaking time. Using a 2 day, 0.33% genipin soak with calf lumbar motion segments, Hedman et al [55] measured a 41% decrease in the neutral zone length. More recently, Barbir et al [11] used a 12 hour soak in 1% genipin with rat discs and found a 58% decrease in tension - compression neutral zone length and a 965% increase in tension - compression neutral zone stiffness. Compared to the present study, the highest genipin treatment produced a 26.3% decrease in neutral zone length and a 242% increase in neutral zone stiffness, both of which are appreciably less than the changes observed with soaking as reported by Hedman and Barbir . Therefore, bilateral, 0.5mL, 400mM genipin injections produce significant and substantial changes, but not as to the same degree that would be expected from soaking.

Unlike the previously cited works by Barbir and Hedman, we used an injection delivery method to simulate a clinically relevant delivery mode. Delivering larger amounts of genipin than are soluble in the buffer solution alone would necessitate larger fluid volumes, and therefore injection pressures, that would potentially damage the tissue. Thus, to achieve amounts of genipin that would be analogous to the previous soaking studies, DMSO was used to increase the genipin solubility in the buffer solution. Unfortunately, for both the 80mM and 400mM conditions, the addition of the DMSO to maintain solubility of the genipin makes it impossible to determine if the observed changes are strictly due to increased crosslinking with genipin, due to the DMSO, or

from an interaction between genipin and DMSO. However, we know of no data suggesting that DMSO may act to increase stability of the disc.

Additional limitations to this study would include the use of bovine tail discs which may not be an appropriate model for painful human discs. The human disc data by Hedman et al [55] showed a larger effect of crosslinking in discs that had larger pre-treatment instability. It is possible that the effects demonstrated in this study may be similarly magnified in unstable human discs. Nevertheless, given that the prevalence of low back pain is ~15% for 18-34 year olds [114] and that there is an increase in reports of adolescent low back pain among Chinese school children [149], studying nondegenerated discs has merit. Additionally, because nondegenerated human cadaveric disc tissue is scarce, bovine tissue presents a practical and lower variability alternative model.

The chosen method of loading in this study, an applied anterior – posterior force to produce a bending moment with no independent compression component was a limitation associated with not having a loading system with multi-axis independent loading capability. An alternative loading method, using the same single axis loading system, would have been to apply the force eccentrically in the superior – inferior direction. That method would have required either repositioning the specimen between flexion-compression and extension-compression loading or alternating the applied force between compression (to produce flexion) and tension (to produce extension). Repositioning the specimen would prevent continuous cycling between flexion and

extension making identification and characterization of the neutral zone more difficult. Changing the load between compression and tension would result in asymmetric loading between flexion and extension. Since shear forces on spinal segments have been linked to instability and pain [85,90,113], the presence of shear and compression forces coupled with flexion-extension moment in our chosen loading method is not unrealistic, though with the moment arm used in this application, the forces were small in comparison with the moment.

Finally, this study was limited to joint flexion – extension neutral zone characteristics. It is possible that other tissue and joint mechanical properties may not exhibit the same uniform response to increasing crosslinking concentration.

3.5. Conclusion

Injection of the disc annulus with increasing concentrations of genipin resulted in corresponding changes in flexion - extension neutral zone metrics as measured by the neutral zone slope, neutral zone length, range of motion, and instability score. Bilateral 0.5mL injections of 20mM genipin did not result in a significant increase in stability for any of the metrics. While 40mM injections produced significant changes in neutral zone stiffness and instability score, 80mM injections were needed to produce significant changes to neutral zone length and ROM. This study establishes the efficacy of injection delivery to affect disc joint mechanics and maps the dose response between injected

genipin and the flexion – extension neutral zone of the disc as characterized by neutral zone stiffness, neutral zone length, instability score, and the segment range of motion.

4. CHANGES IN DEGRADATION RESISTANCE OF DISC ANNULUS FIBROSUS AFTER CROSSLINKING

4.1. Introduction

Proposed pathways describing degradation of the intervertebral disc (IVD) have included, but are not limited to, mechanical, biochemical, and genetic [3,4,12,19,51,68,81]. While these pathways have been widely reported on, few studies have combined multiple pathways to delineate their relative contributions as singular components or the contributions of the interactions between these mechanisms.

The effects of biochemical degradation have been previously noted for collagenous tissues such as pericardia, bioprosthetic heart valves (BHVs), and articular cartilage [34,60,101,112,115,117]. With respect to the IVD specifically, Barbir [11] demonstrated that collagenase and elastase significantly altered rat motion segment stiffness and neutral zone length after soak treatments and Kuo [75] described changes in the stiffness and damping coefficient of porcine motion segments after trypsin soak. Isaacs [64] reported on significant changes to annulus tissue mechanical properties such as UTS, elastic modulus, and energy after soaking in collagenase, elastase, and Chondroitinase ABC. Further, the presence of collagenase, elastase, matrix metalloproteinases (MMPs), and trypsin have been demonstrated to correlate with in vivo disc degradation, tears [6,68,143], and herniation of the nucleus [53,84].

Similar to biochemical degradation, degradation from cyclic mechanical loading of collagenous tissues has been the subject of numerous studies for cartilage [25,96,125], BHVs [101,138], and the IVD. For the IVD, cyclic mechanical loading has been shown to alter motion segment height [65,78,79,134], failure stress [46,52,63], and stiffness [3,75]. Callaghan [17] demonstrated that repetitive flexion-extension of motion segments combined with axial loading induced herniations in human motion segments.

The use of exogenous crosslinking to modulate tissue properties has been described for use in engineering scaffolds [76], BHVs [7], knee meniscus allografts [62], and to augment weakened disc annulus [23,103,108]. Exogenous formed crosslinks have been demonstrated to reduce degradation from enzymatic activity as well as alter tissue mechanical behavior. During biochemical degradation of collagen, collagenolytic enzymes act on the peptide bonds to cleave the molecule into smaller units. By preventing penetration into the matrix, and supporting cleaved chains, crosslinked tissue can better resist biochemical degradation as well as the decreases in mechanical properties associated with biochemical degradation[92]. Mi [87] reported increased fixation indices in the knee meniscus after crosslinking treatments. Resistance to degradation of pericardia with crosslinking was demonstrated by maintenance of denaturation temperature and ultimate tensile strength up to 12 weeks after subcutaneous implantation [60] and after soaking in collagenase [117]. In the rat intervertebral disc, Yeramalli [150] demonstrated that enzymatic loading decreased tension modulus, compression modulus, and neutral zone modulus, while increasing neutral zone length. In contrast, genipin crosslinking of the nucleus

produced the opposite result but the genipin results were not statistically significant and control injections alone produced significant differences. Similarly Kuo [75] reported that biochemical degradation of porcine motion segments with trypsin decreased the disc's dynamic properties, while crosslinking treatment of trypsin degraded discs helped resist fatigue related changes in the disc's dynamic properties. However, differences in total injection volume between treatments make comparison of the effect sizes difficult as mechanical behavior of the motion segment is related to its hydration [1,56,83,89,149].

In addition to increased resistance to biochemical induced degradation, crosslinking may also afford increased resistance to mechanically induced degradation due to the increased connectivity of the collagen fibers which help maintain fiber alignment as well as increase the number of physical bonds to distribute the loading. BHVs demonstrate increased mechanical performance with increased crosslinking [7,101]. At the tissue level, increases in mechanical properties from crosslinking [23,108,117] increase load carrying ability and may translate to increased fatigue resistance. To this point, Kuo[75] suggested that "dynamic properties of the denatured discs are better recovered by the elevated crosslinking", but pre- to post-fatigue comparisons are confounded because there was no provision for recovery of the expelled fluid and motion segment stiffness is related to hydration [1,56,83,89,148].

In this study, the changes to the mechanical behavior of bovine disc annulus samples after biochemical degradation, cyclic mechanical compression, and crosslinking

treatment with genipin are demonstrated. We hypothesized that biochemical and mechanical degradation would alter tissue geometry and compliance and that crosslinking treatment would attenuate the observed degradation. Further, we hypothesized that biochemical and cyclic mechanical loading may interact and produce changes that are not obvious from isolated study of the degradation factors.

4.2. Methods

Two experiments were performed using different material models. The first experiment used cast gelatin in order to provide highly uniform samples and to ameliorate the effect of fluid flow through the specimen. The second experiment used cylindrical punched specimens taken from the annulus of fresh frozen bovine tail discs.

4.2.1. Experiment I: Gelatin

Gelatin (Sigma G9382-100G) was mixed 100 mg per ml with 50mM EPPS Phosphate buffer ph 9 and poured into a mold to cast 20 cylinders with dimensions of 9.6 mm dia. X 7.9 mm tall. Crosslinking treatment was done on 10 specimens by soaking specimens in 20mM GP with 50mM EPPS Phosphate buffer (ph9) at room temperature for 4 hours. The other 10 specimens were soaked in buffer only (sham soak) at room temperature for 4 hours. All specimens were soaked overnight at 4C in filtered water and then for 1 hour at test temperature before testing.

Gelatin specimens were subjected to degradation by hydrolysis, thermal, and cyclic mechanical loadings. Tests were conducted in a temperature controlled PBS bath, with

crosslinked gelatin samples tested at 37 °C and control samples (s) tested at room temperature after pilot testing demonstrated that the sham treated samples degraded too quickly at 37 °C for testing.

Mechanical degradation of the samples was done using a cyclical compressive load applied as a triangular wave form from 0.02N to 0.2 N at a defined strain rate of 1 mm/sec. At predetermined intervals, characterization of the mechanical properties was performed with a ramped compressive load (0.1 mm/s) to peak force of 0.2 N followed by returning to start position. From the ramped loading and unloading, specimen height, compliance, and hysteresis were calculated. The mass of the gelatin samples was also measured at each interval and used to calculate the swell ratio.

Crosslinked gelatin samples were evaluated at 0, 500, 1000, 2000, 4000, 6000, and 8000 cycles. Control samples were only evaluated at 0, 500, and 1000 cycles after which they were not suitable for further testing.

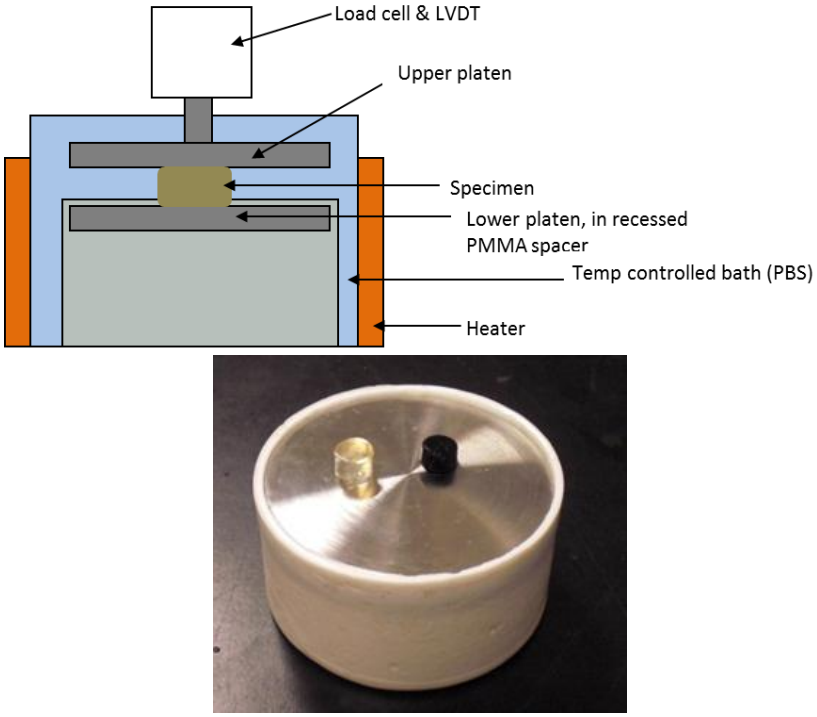

Figure 14. Schematic of the unconfined compression test (top) and photograph of untreated and treated gelatin specimens (bottom).

4.2.2. Experiment II: Disc Annular Tissue

Fresh frozen bovine tail discs were removed from the vertebrae, soaked overnight in PBS at 4 °C and frozen. Samples of the annulus fibrosus were punched using a 7.9mm diameter punch while still frozen. Each sample appeared to contain approximately 6 to 12 lamella. If needed, the loading surfaces of the cylindrical samples were hand trimmed using a scalpel to maintain parallelism.

Annulus samples were subjected to degradation by biochemical (enzymatic) and repetitive mechanical loadings as well as matrix augmentation by genipin crosslinking. There were five treatment conditions: control, genipin only, trypsin only, trypsin followed by genipin, and genipin followed by trypsin. Control specimens were soaked 4 hours in 50mM EPPS Phosphate buffer (ph9) at 37°C. Genipin only specimens were soaked 4 hours in 50mM EPPS Phosphate (ph9) with 20mM genipin at 37°C. Trypsin only specimens were injected with 60 μL of 0.5% Tryspin through a 28 gage needle and then soaked 4 hours in the same manner as controls. The specimens in these three treatment groups were then stored overnight in their respective solutions at 4°C. Trypsin followed by genipin samples were injected and soaked overnight in the same manner as the trypsin only specimens, stored overnight at 4°C, then treated with genipin in the same manner as the genipin only specimens and stored overnight at 4°C. Genipin followed by trypsin specimens were genipin soaked in the same manner as the genipin only specimens, stored overnight at 4°, then treated with trypsin in the same manner as the trypsin only samples and stored overnight at 4°C.

Tests were conducted in a 23°C PBS bath after allowing 1 hour for specimen temperature to stabilize. The characterization of mechanical properties consisted of applying a ramped load at 0.1 mm/s up to 4N and then immediately returning to start position. Between evaluations, samples were cyclically loaded using a triangular wave form from 0.4N to 4 N at a constant load rate which varied to maintain the overall cycling frequency of about 4 Hz. Specimens were loaded 10,000 cycles and then soaked unloaded in PBS overnight at 4°C. Property characterization was done at 0, 5000, 10000 cycles, and after overnight soaking.

4.2.3. Data Analysis

The data collected during the evaluation protocols was analyzed to determine the specimen height, hysteresis, and compliance. Specimen height was determined from the platen height at the tare load during the evaluation ramp loading. Because the disc annulus specimens demonstrated initial differences in height due to differences in the size of the disc from which they were obtained, normalized annulus height was calculated as the ratio to pre cycled height. For the gelatin specimens, secant compliance was calculated as the change in height during the evaluation cycle divided by the peak force. For the disc annulus specimens, because of the very large changes in height that occurred during cycling, referential secant compliance was calculated using the loaded height minus the pre cycled unloaded height and then normalizing by the pre cycled height. Hysteresis was determined first by integrating the force displacement curve during the loading portion of the first evaluation cycle to determine loading energy

and integrating the unloading portion of the curve to determine the unloading energy. Hysteresis was then calculated as the difference in the loading and unloading energies.

4.2.4. Statistics

The percent change in the mechanical parameters within treatments was calculated and tested using the Wilcoxon Rank Sum nonparametric statistic for difference from the 1st cycle for paired samples at 10,000 cycles and after recovery soak. Differences between treatments at the 1st cycle were tested using the two group Kruskal Wallis nonparametric test. The software package STATA R11 was used for all statistical comparisons and significance was set for $p<0.05$ for both tests. Because of the large number of possible comparisons, statistical significance was only calculated for control, genipin-only, and trypsin-only treatments.

4.3. Results

4.3.1. Gelatin

Crosslinking the gelatin samples produced dramatic changes in the compliance, hysteresis, and swell compared to the control samples (Table 3). Control specimens rapidly degraded and were unable to complete 8,000 cycles even after lowering the bath temperature from 37°C to 27°C. Therefore, the control specimens were only run to 1,000 cycles at which the percent change in height, hysteresis, swell ratio, and secant compliance were all statistically significantly. Only 5 of the 8 control samples were able to be collected to 1,000 cycles. In contrast, the crosslink treated samples demonstrated

few changes even after 8,000 cycles. A statistically significant increase in specimen height of 5.5% was observed after 8,000 cycles (Figure 15).

Table 3 . Properties of the gelatin specimens. * Denotes significant difference after cycling, p<0.05.

		Swell Ratio	Height (mm)	Hysteresis (N*mm)	Secant Compliance (mm/N)
control					
	Initial	1799	6.467	74160	13.40
	after 1,000 cycles	1496*	3.256*	9620*	4.79*
genipin treated					
	initial	988	7.842	5376	0.750
	after 8,000 cycles	1064	8.267*	5065	0.656

4.3.2. Annulus Fibrosus

All treatment groups were successfully tested for 10,000 cycles.

Annulus samples not receiving crosslinking treatment did not retain their initial lamellar appearance (Figure 16) and demonstrated more fluid like behavior after testing. For example, outside of the bath these samples *did not* maintain their shape. Trypsin treated samples demonstrated even greater loss of lamellar structure and increased fluid like behavior. Crosslinked annulus samples generally retained their lamellar appearance and behaved in a more solid-like manner. For example, outside of the bath these samples *did*

maintain their shape. After cycling, specimen diameter subjectively appeared less circular than initially, with elongation of the diameter occurring along an axis normal to the lamella plane. The observed elongation appeared greatest in the trypsin treated specimens, least in the crosslinked specimens and control specimen elongation appeared in between the trypsin and crosslinked specimens.

4.3.3. Height

At 5,000 and 10,000 cycles, all specimens demonstrated very large decreases in unloaded normalized height (Figure 17). Control specimen normalized heights were 0.46 after 5k cycles and 0.40 ($p<.05$) after 10k cycles. Genipin-only specimen normalized heights during cycling were 0.64 at 5k cycles and 0.57 ($p<.05$) at 10k cycles. Relative to control specimens, genipin height was 41.9% larger than controls after 5K cycles and 44.2% larger after 10K cycles. Trypsin-only specimen normalized heights during cycling were 0.24 at 5k cycles and 0.23 ($p<.05$) at 10k cycles. Relative to control specimens, trypsin height was 40.9% smaller than controls after 5K cycles and 35.5% smaller after 10K cycles. Combinations of genipin and trypsin together demonstrated height loss during cycling similar to controls and in between genipin and trypsin specimens. Trypsin followed by genipin treated specimen normalized height relative to controls was 11.5% smaller after 5K cycles and 7.1% smaller after 10K cycles. Genipin followed by trypsin treated specimen normalized height relative to control samples was 6.2% larger after 5K cycles and 13.5% larger after 10K cycles.

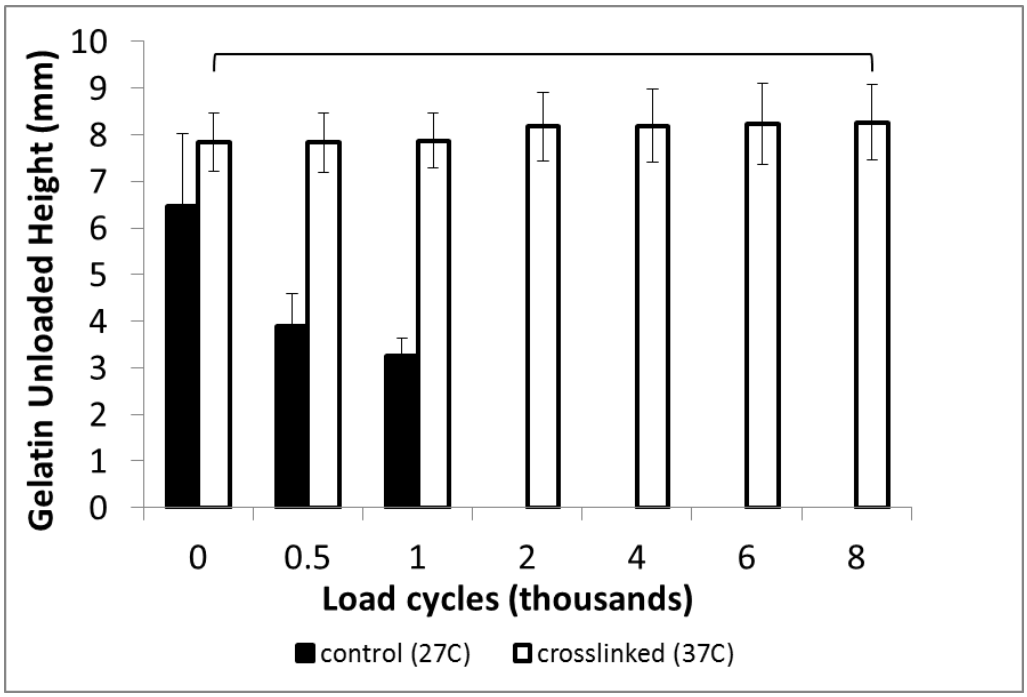

Figure 15 Change in Specimen height of gelatin samples with cyclic mechanical loading.

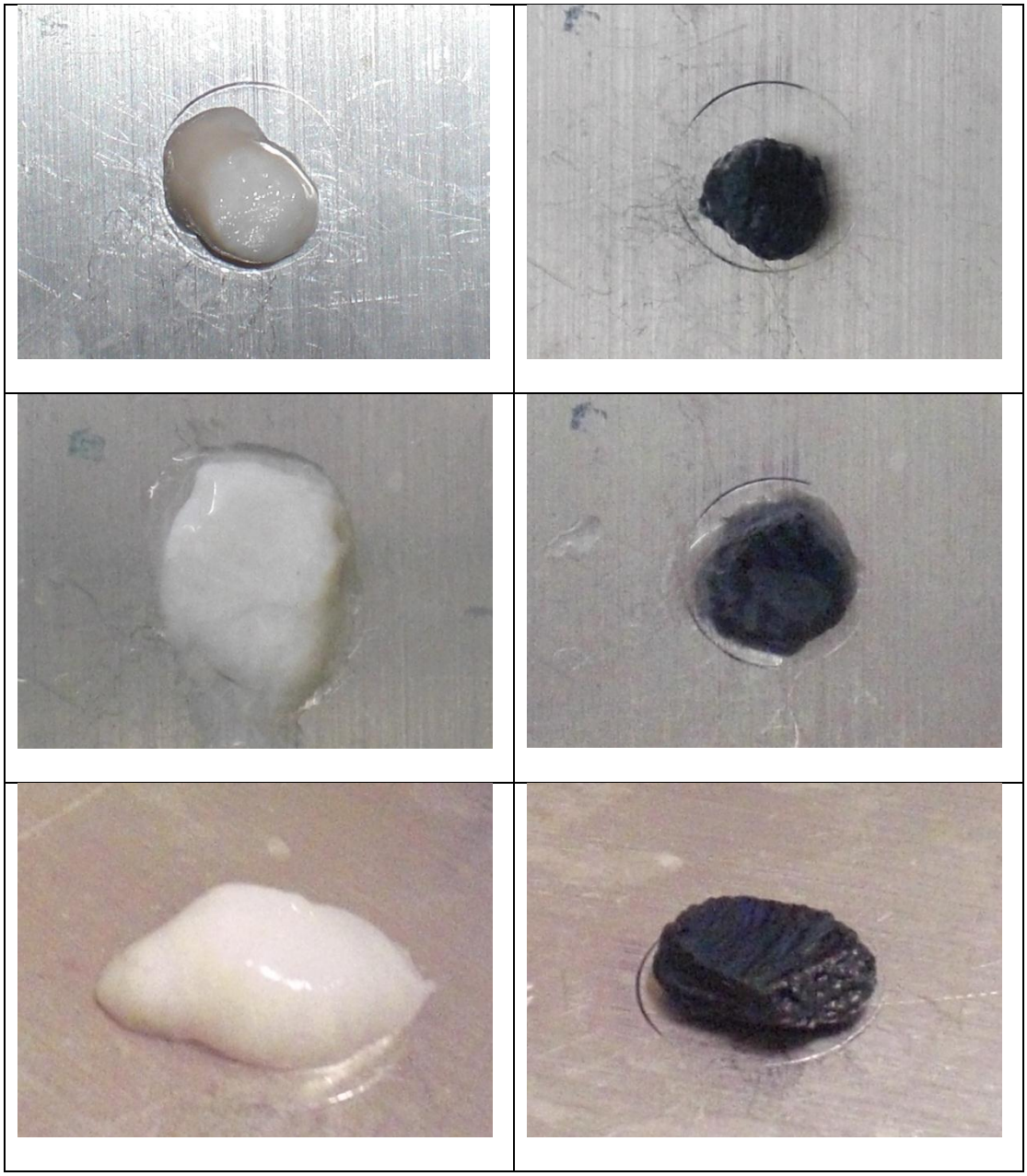

Figure 16. Example of specimens receiving trypsin treatment (left column) and genipin crosslink treatment (right column), before mechanical cycling (top row), and after 10,000 cycles with recovery soak (middle and bottom rows).

After overnight soaking, control specimen normalized height was 0.91. For genipin treated samples, normalized height after soak was 0.81 ($p<0.05$ compared to initial height) and was 10.3% less than control post soak height. For trypsin treated specimens, normalized height after soak was 0.98, 7.3% larger than the control post soak height. Both combination treatments exhibited marked lack of recovery, with a normalized height after soak of 0.51 for both treatments, 44% less than control post soak height.

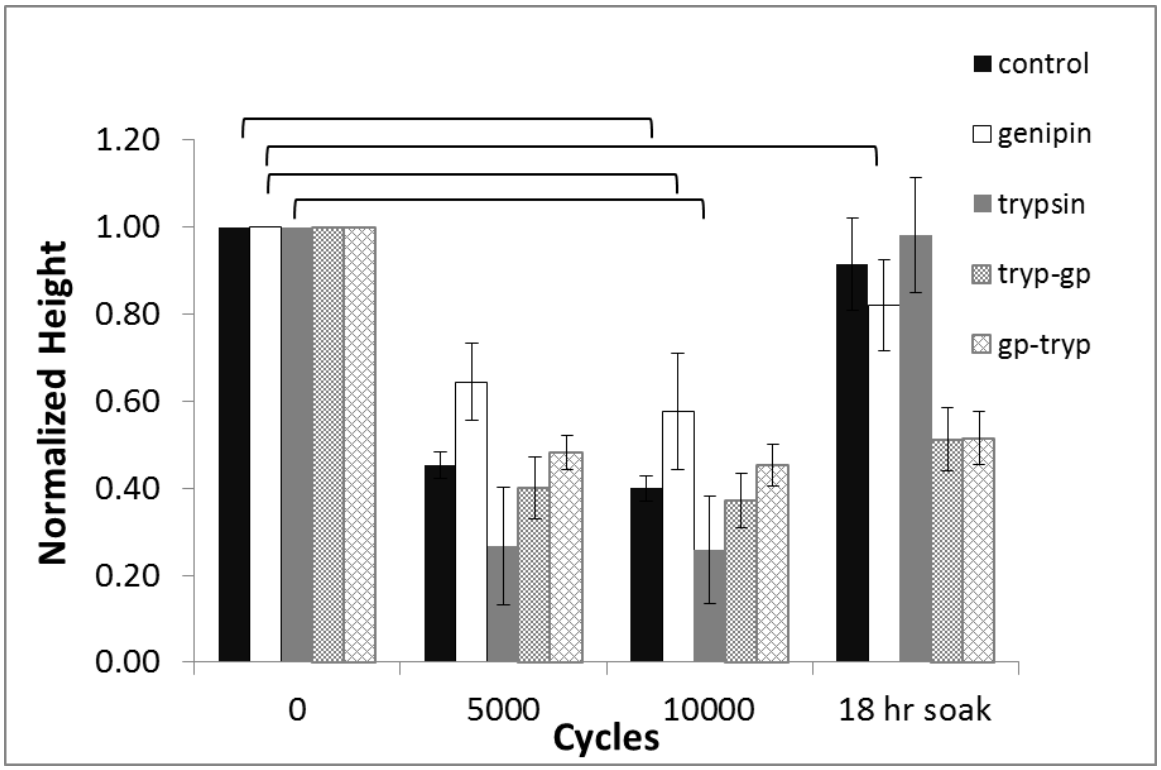

Figure 17. Effect of mechanical and enzymatic degradation on annulus normalized height.

4.3.4. Compressive Compliance

At 5,000 and 10,000 cycles, all specimens demonstrated increases in secant compressive compliance (Figure 18). Control specimen compliance was 0.105 mm/N initially, 0.188 mm/N after 5k cycles, and 0.200 mm/N ($p<.05$) after 10k cycles. Genipin-only compliance during cycling was .094 mm/N initially, 0.140 mm/N at 5k cycles, and 0.157 mm/N ($p<.05$) at 10k cycles. Relative to control samples, genipin sample compliance started 10.8% lower and was 25.6% lower after 5k cycles and 21.7% lower after 10k cycles. Trypsin-only compliance during cycling was 0.166 mm/N initially, 0.239 mm/N at 5k cycles, and 0.245 mm/N ($p<.05$) at 10k cycles. Relative to control samples, trypsin sample compliance started 58.1% higher ($p<0.05$) and changed to 26.9% higher after 5k cycles and 22.8% higher after 10k cycles. Combinations of genipin and trypsin together demonstrated compliance loss during cycling in between that of genipin only and trypsin treated specimens. Trypsin followed by genipin treated specimen compliance started 42.6% higher than control specimens and changed to 3.7% larger after 5k cycles and 0.1% lower after 10k cycles. Genipin followed by trypsin treated specimen compliance started 12.2% larger than control specimens and changed to 8.8% lower after 5k cycles and 11.6% lower after 10k cycles.

After overnight soaking (Figure 18), control specimen compliance was 0.116 mm/N. Genipin post soak compliance was 0.119 mm/N, 2.5% larger than post soak control compliance. Trypsin post soak compliance was 0.175 mm/N, 51.1% lower than post soak control compliance. Combination treatments of trypsin and genipin had post soak

compliance values of 0.168 mm/N and 0.166 mm/N and were 45.1% and 43.4% larger than control post soak compliance.

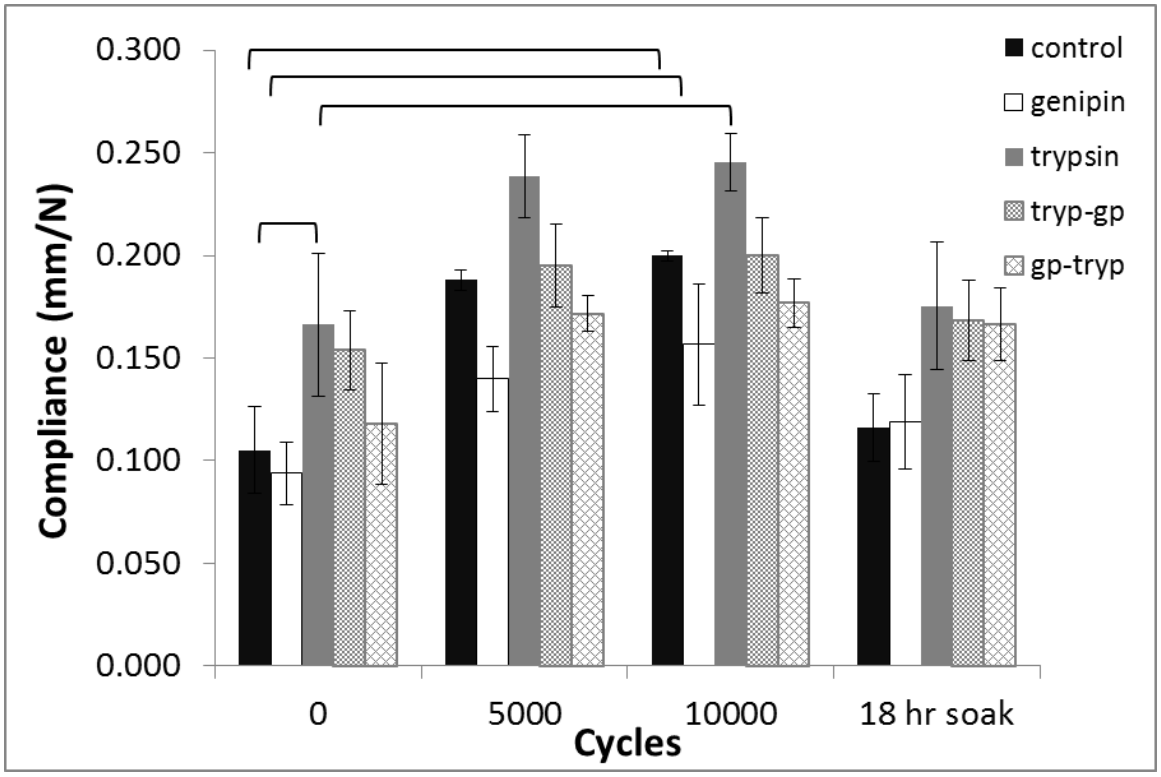

Figure 18. Effect of mechanical and enzymatic degradation on annulus compliance (mm/N).

4.4. Discussion

Effects to the mechanical behavior of both collagen based gelatin and bovine disc annulus after biochemical degradation, cyclic mechanical compression, and crosslinking treatment with genipin were studied. Gelatin samples treated with genipin crosslinking were observed to have greatly increased resistance to degradation, with only minor

degradation occurring after 8,000 cycles. Annulus samples experienced large changes in geometry and compliance consistent with visual observations of induced degradation during cycling. Biochemical degradation with trypsin increased the magnitude of changes, while crosslinking with genipin resisted changes. Changes to height and compliance during cycling appeared to be mostly recovered after unloaded, overnight soaking.

4.4.1. Gelatin Specimens

In contrast to control samples, crosslink treated gelatin samples were successfully loaded in cyclic compression for 8,000 cycles at 37 °C, with the only detectable change being a 5.5% increase in sample height. Since this increase in height was accompanied by an increase in swell ratio of 2% (not significant) and is consistent with observations of increased swell in articular cartilage after mechanical degradation Bank [8], it seems likely that the samples experienced some matrix degradation from the mechanical cyclic compressive loading. Interpolating the 5.5% height change observed in the crosslinked specimens to the control specimens suggests the controls would have reached the same percent change in height in 69 cycles; thus, the crosslinking treatment increased the degradation resistance of the gelatin samples more than 100-fold even without considering the 10°C drop in bath temperature.

4.4.2. Annulus Specimens

4.4.2.1. Effects of Biochemical Treatments Before Cyclic Degradation

For genipin-only treatments, there was no significant difference in the initial axial compressive secant compliance compared to controls while trypsin-only treated

specimens did demonstrate a significantly increased compliance 58% larger than the controls. Trypsin was previously shown by Kuo [75] to decrease dynamic stiffness modulus of the disc. Crosslinking has shown mixed results to affect various stiffness measurements. Slusaraewicz [108] showed a significant increase in circumferential tensile modulus after treatment with MG, but no significant difference after treatment with GP. Yeramalli [150] showed no significant difference in axial tension or compression modulus. Chuang [23] reported significant differences in high stiffness modulus in circumferential tension, but not high stiffness axial compression

Treatment levels of genipin and trypsin were taken from literature reports demonstrating differences in tissue behavior, and there were observable differences between treatments with cycling. The overnight soakings clearly influence hydration (which therefore alters the mechanical behavior) and were implemented first to assure that enough genipin could enter the matrix to effectively crosslink the entire matrix and second to minimize transient swelling effects that would have occurred if the specimens were placed in the bath for testing without first approaching hydration equilibrium. As both crosslinking and chemical degradation have been shown to influence tissue hydration [16,26,97,128,148] it is possible that differences in initial compliance after genipin treatment were overshadowed due to changes in hydration that accompanied the crosslinking treatment.

4.4.2.2. Effects of Biochemical Degradation During Cyclic Compression

Cyclic mechanical compression of the control specimens produced a significant reduction in normalized height and increased compliance relative to initial values. Additionally, subjective observations of the specimen shape were that the specimen diameter tended to distort along an axis normal to the lamella plane and is consistent with observations reported by Urban [132] regarding the anisotropy of annulus swelling. The visual appearance also suggested decreased structure of the lamellae and fibers.

All specimens, regardless of treatment, showed decreased height with cycling. Genipin treated specimens showed less loss of height compared to controls and visually appeared to better resist degradation during cycling as the structure of the lamella was intact after cycling. Resistance to height loss was consistent with both increased matrix integrity and increased fluid retention (relative to controls), and both are effects consistent with changes that have been reported previously for crosslinked tissue [11,16,108,128,140] . It was interesting to note that the difference in height of the genipin treated specimens persisted for all 10,000 cycles.

Trypsin treated specimens showed greater loss of height compared to controls and visually appeared to degrade more with mechanical cycling as indicated by the loss of lamella structure and increase in amorphous appearance. Increased height loss was consistent with both decreased matrix integrity and decreased fluid retention, both

effects consistent with changes that have been reported previously for chemically degraded tissue [75,97,148].

Although the behavior of the genipin treated samples during cycling suggests higher fluid retention than controls and the behavior of trypsin treated samples suggests less fluid retention, we did not specifically measure water content of the specimens, nor the degree to which loading-induced loss of matrix integrity (observed visually) would contribute to the loss of fluid with continued cyclic loading. Any loading-induced matrix degradation would be expected to allow a greater imbibition of water during the final unloaded soaking, such that the near-initial height and compliance following the final free-soak could be the result of hyper-hydration of the cycled tissue. Figure 3 and similar visual observations appear to confirm this hyper-hydration and increase in volume in the control and typsin treated specimens after post-cycling re-soaking.

Further, without measurements of hydraulic permeability or proteoglycan concentration there can be no delineation as to the contribution of the mechanisms by which genipin or trypsin treated samples exhibited changes in fluid retention compared to controls. Combinations of trypsin and genipin resulted in similar height and compliance changes during cycling as was seen in the control specimens suggesting that the crosslink augmentation effect approximately balanced the enzymatic degradation effect. However, for combination treatments, the change in compliance after recovery was more

similar to the effect seen in the trypsin only treatment than in the controls or genipin treatments.

4.4.2.3. Effects of Biochemical Treatments after Cyclic Degradation and Recovery Soak

Control specimen normalized height recovered to 90% of initial height after unloaded soaking and compliance remained 10.4% larger than initial, though neither change was significantly different from the initial control specimen values. If the annulus specimens degraded like the gelatin specimens, a slight increase in specimen height after the recovery soak would be expected. However, the large changes in height during cycling as well as visual observations of the large changes in diameter suggest the annulus specimens behaved differently than the gelatin specimens. Other studies have noted loss of disc height [78,79] and others increased stiffness [65] during compression testing of the IVD. Conversely, Iatridis [63] reported that cyclic *tension* of annulus fibrosus produced a significant increase of 9% in recovered, normalized length. Compression testing may be less sensitive at detecting degradation induced matrix expansion in the axial direction because the compressive load acts to deform the matrix in the opposite direction of the degradation induced expansion and the resulting loss of lamella structure into a more amorphous shape consistent with radial expansion that may have been at the expense of specimen height.

After soaking, trypsin group specimen height recovered to 95% of initial height, which was 6% more than the height recovery of controls, suggesting greater fluid reabsorption

than in the controls. Such an effect is consistent with increased hydraulic permeability allowing more fluid to re-enter the matrix during the soak, and consistent with a general loss of matrix integrity that decreases fiber tension, decreases fiber alignment, and allows expansion and distortion of the matrix, both of which were suggested as the mechanisms for changes in specimen height during cycling. Further, trypsin specimen compliance remained 5.5% larger than the trypsin initial compliance, but this was not significant.

After soaking, genipin treatment group specimens only recovered 81% of their initial height, which was significant and also 10% less than control specimen height recovery, suggesting less fluid reabsorption in the genipin treated specimens than in controls. Other studies have reported increased permeability and proteoglycan retention with genipin treatment [24,128] suggesting the genipin specimens would have absorbed as much or more fluid than controls, but with no measurements of hydraulic permeability or proteoglycan concentration or matrix degradation of the controls there can be no confirmation of whether these parameters were altered in this study or how they may have affected recovery. One explanation for this difference in fluid absorption could be less resistance to fluid inflow in controls due to somewhat greater cycling induced degradation and perhaps compaction of the matrix compared to genipin treated specimens. Another possible explanation for a difference is that the genipin samples continued to form crosslinks during the cycling and that this additional crosslinking restricted the matrix geometry from returning to the initial height.

For combination genipin-trypsin treatments, the recovery soak was much less effective at restoring height and compliance with large differences remaining for both combination treatments. Because all three treatments containing genipin displayed lack of height recovery, we considered that there could have been additional crosslinking that occurred while the specimen was deformed from the cycling. Sources of additional crosslinking activity could arise from unreacted crosslinking agent that remained in the matrix or from incomplete crosslink chains. As loading altered the matrix geometry the distances between available bonding sites could have decreased to allow further crosslinking of the incomplete chains, or as the matrix denatured under the cyclic loading [25] access to new crosslinking sites could have been exposed. Treatments including trypsin with genipin would be expected to have greater additional crosslinking since the trypsin degradation would have exposed even more potential crosslinking sites and weaken the matrix allowing greater deformation. In fact, the two genipin-trypsin combinations did show the greatest unrecoverable height loss.

4.4.2.4. Potential of Additional Crosslinking During Cycling

To test if additional crosslinking may have formed after the initial crosslinking soak, two additional trypsin + genipin samples were prepared and then treated with a soak in excess glycine. The glycine was used to attach to potential crosslinking sites in order to limit additional crosslink formation. The percentage of recovered height for the two glycine treated specimens was 63% which was greater than the 51% height recovery observed in the trypsin + genipin treated samples without glycine. The 12% increased

height recovery achieved after the glycine treatment is quite similar to the 10% difference in control specimens and the genipin only treatment group which supports the hypothesis that glycine may have blocked further crosslinking. However, it does not appear to account for all of the unrecovered height in the combination treatments.

4.5. Conclusion

Effects to the mechanical behavior of bovine disc annulus after biochemical degradation, cyclic mechanical compression, and crosslinking treatment with genipin were studied. Accelerated cyclic loading to 10,000 cycles with and without biochemical treatments produced large changes in specimen height and compliance during cycling. Both height and axial secant compliance of mechanically cycled but not biochemically treated samples recovered to 10% of initial values after unloaded, overnight soaking. Compressive loading with biochemical degradation by trypsin was observed to alter the mechanical behavior under compressive loading producing more severe loss of height, and increased compliance, relative to control specimens. With genipin crosslinking, compressive loading produced less loss of height and less increase compliance relative to control specimens. Recovery of genipin specimen height and compliance was hampered, possibly by a continuation of crosslinking that constrained matrix expansion and restricted fluid inflow.

5. CHANGES IN THE INTERFACIAL SHEAR RESISTANCE OF DISC ANNULUS FIBROSUS FROM GENIPIN CROSSLINKING

5.1. Introduction

The gross anatomy of the annulus fibrosis has been classically described to consist of concentric lamella composed of parallel collagen fibers with orientation that alters from adjacent lamella [28]. Tear like defects in the annulus fibrosis of the intervertebral disc are classified usually by location and direction of the tear, as in rim lesion (RL), concentric tear (CT), or radiating tear (RT) [136]. Such tears are common in adult human discs and are strongly correlated to age, with CT tears, comprised primarily of delamination of adjacent lamellae, being the first to appear and the most common [93,136].

Mechanically, annular tears are known to affect the behavior of the annulus and motion segment. Fazzalari [36] described decreased stiffness in lateral bending and flexion with induced CT using a sheep model. Thompson [126] reported that human cadaver motion segments containing disc lesions had decreased torsional stiffness compared to lesion free discs, but the 'with lesion' group was older than the 'lesion free' group. He further described positive correlations between CT and flexion – extension stiffness. In addition to affecting mechanics, CT tears may affect other types of tears due to increased stress near the structural defect [44,93].

A direct connection between annular tears and pain has only been weakly established. In Thompson's study all cadaver donors were described as having "no history of back pain" and Fazzalari's study utilized animals. Osti [93] postulated that pain provocation during discography was "closely related to the presence of tears extending to the outer lamellae of the annulus fibrosus". Freemont [39] demonstrated that painful discs had more sensory innervation and deeper innervation than pain free discs and aged and pain free discs. The presence of sensory innervation near a lesion where shearing stresses are increased due to the structure defect [44] seems a reasonable mechanism for pain generation but has yet to be proven true. Recently Weiler [143] discussed a connection between annulus tearing/degradation and the presence and transport of matrix metalloproteinases that are linked with degenerated discs and cytokines that that may cause or modulate pain. Nevertheless, the presence of annulus tears (or the remaining scar) is evidence of structural overload, and the tear itself presents a structural failure thereby meeting Adams [4] proposed definition for a degenerated disc.

The use of exogenous crosslinking to modulate tissue and biomaterial properties has been described for use in engineering scaffolds [76], bioprosthetic heart valves [7], knee meniscus [62], and to the weakened intervertebral disc [23,55,103] . At the tissue level, increases in mechanical properties from crosslinking [108,117] increase load carrying ability and elongation properties of tissue and may therefore directly influence the mechanisms involved in tearing.

Recently, Gregory [47] described a method of testing the interface strength between disc lamella, observing that the shear stresses that lead to de-bonding are concentrated at the edge of the bonded interface. The described test presents a useful tool for understanding interlamellar tears and their propagation. The primary objective of the present study was to test if crosslinking treatment of disc annulus would increase the force needed to disrupt the interlamellar interface using a method similar to the one described by Gregory. In addition to crosslinking increasing the disruption force, we hypothesized that crosslinking would increase the stiffness of the lamella interface and the work needed to achieve disruption. If successful, exogenous crosslinking could present as a therapy to minimize or prevent annular tears, assisting in the treatment of degenerated disc disease and back pain.

5.2. Methods

Three fresh frozen, skinned bovine tails were obtained from a local supplier. The adjacent musculature was removed, and the spines stored wrapped in plastic. While still frozen, the caudal disc locations were identified, cut along the mid sagittal plane, and then excised from the endplate with a straight razor. The nucleus was removed and the annulus samples placed in 0.15M PBS to soak overnight at 4°C. The overnight free swelling enlarged the lamella making identification and dissection of the lamella interface easier. After swelling, specimens were frozen and then while still frozen hand-dissected using a scalpel to isolate a single inter lamella interface (Figure 19), from the outer half of the annulus. Additional adjacent lamellas were maintained on both sides of

the interlamellar interface of the specimen to provide increased strength at the clamp site. Samples were then soaked for 4 hours at 37°C in 50mM EPPS Phosphate buffer (ph9) (sham) or 50mM EPPS Phosphate (ph9) with 20mM genipin at 37°C (crosslink treated) in order to provide sufficient and uniform delivery of genipin. After treatment, specimens were stored overnight at 4C in their respective solutions.

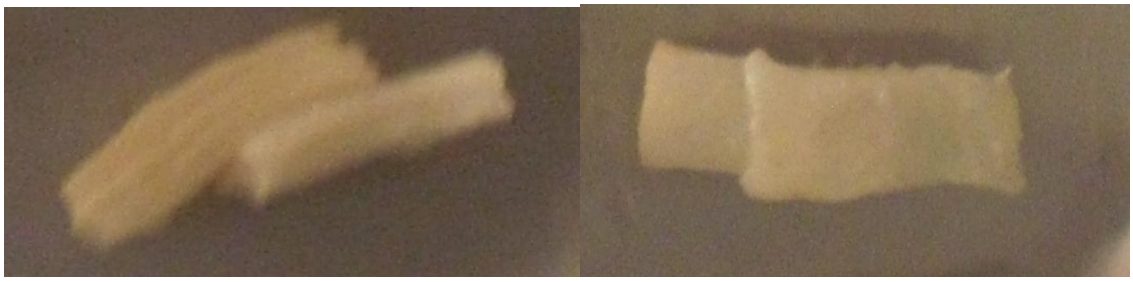

Figure 19. Bovine annulus specimens after dissection to isolate a single interlamellar interface. Note presence of multiple lamella on either side of interface (left photo).

One disc per tail was successfully dissected into four sections and each of the four sections was successfully prepared producing 12 specimens from the 3 tails. A second disc was taken from one tail and three samples were successfully prepared resulting in a total of 15 samples. To reduce inter-specimen variability, samples were paired so that there would be two sham and two genipin treated samples for each disc, and each sham/genipin pair was further grouped by size. Of the 15 samples prepared, all were tested, but three failed at the clamp site and their data was not included. It is possible that these samples may have been improperly dissected and contained a lamella that bridged between the clamping sites.

Mechanical testing was conducted using a Test Resources R-1000 frame with a 100N load cell. Custom manufactured rake-like fixtures were used to clamp the specimens during testing (Figure 20). The location of the interlamellar boundaries were marked with a gel pen on the lamina plane and the location of the interface midpoint was indicated with a gel pen on the side of the specimen. Two digital video cameras monitored the specimens during testing. From the digital video image, the stretch of the interlamellar interface was calculated from the position of the boundary marks. The force - displacement output of the test machine was synchronized with the interlamellar interface stretch measured from the video by tracking the clamp positions which were visible in the digital video. Specimen interface width, thickness and length were measured using calipers immediately before testing and force was divided by interface width to normalize for specimen differences and to be consistent with the analysis presented by Gregory. Three preconditioning cycles were performed at 1%/sec to 10% strain and testing was carried out at 2%/sec to be consistent with Gregory [47].

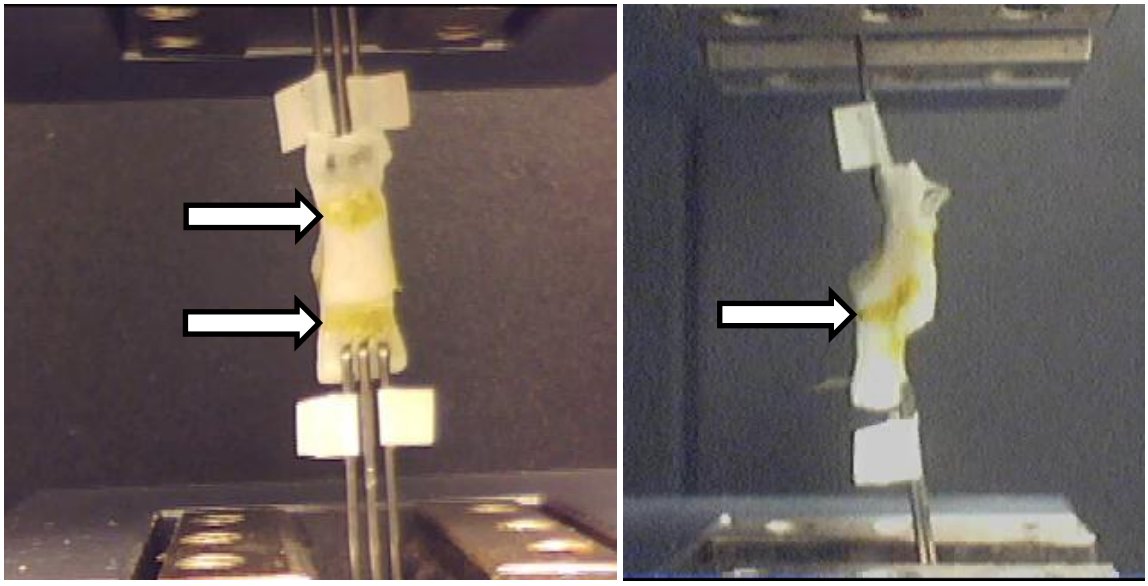

Figure 20. Lamellar interface plane view (left) showing marked interface boundaries (arrows) and side view (right) with mark indicating midpoint of interface.

Using custom Octave scripts (Figure 21), stiffness was calculated by fitting a line through the linear region of the force (normalized by width) – displacement curve. Yield force was taken at the point where there was a deviation from the linear fit of 2%. Peak force was taken as the largest force from the test. Work was calculated as the area under the force - displacement curve up to yield and to peak as appropriate. Statistical significance was tested using the Wilcoxon Rank Sum test on the paired differences described for disc and size. The Stata R11 software package was used for all statistical comparisons.

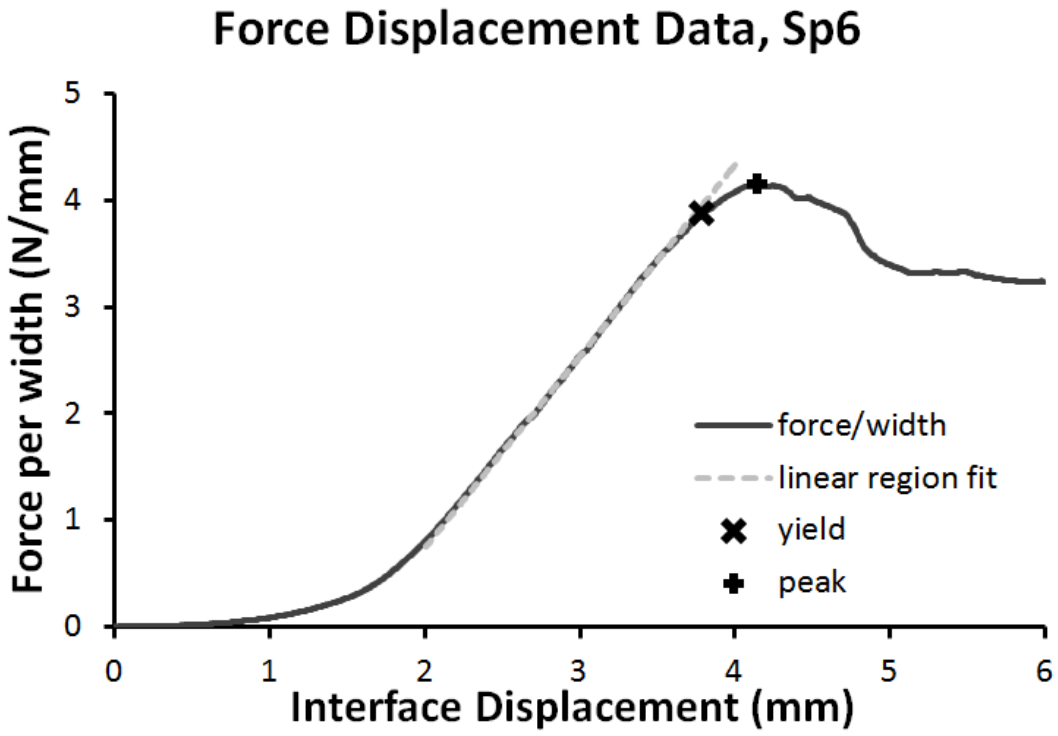

Figure 21. Example of the force displacement data with the yield point, peak point, and linear region stiffness.

5.3. Results

Fifteen specimens were prepared for testing, but only twelve were successfully tested. Of the successfully tested specimens, six were sham treated and six were genipin treated. Two of the three specimens that were not successfully tested failed at the clamp site and one of the specimens continued to slip off the rake without failing in the interlamellar interface. For the specimens whose preplanned paired counterpart was not successfully tested, the specimen was paired with the other treatment specimen from the same disc.

Table 4 summarizes the data. The mean yield force per width was 2.43 N/mm for sham treated specimens, 3.87 N/mm for genipin treated specimens (59% greater than sham specimens), and the paired difference between the groups was 1.81 N/mm which was statistically significant ($p<.018$). Figure 22 presents the yield force and peak force per width data graphically. The mean peak force per width was 2.65 N/mm for sham treated specimens, 4.50 N/mm for genipin treated specimens (70% greater than sham specimens), and the paired difference between the groups was 2.23 N/mm which was statistically significant ($p<.018$). The mean work to yield was 2.16 J/mm for sham treated specimens, 3.65 J/mm for genipin treated specimens (69% greater), and the paired difference between the groups was 2.38 J/mm which was statistically significant ($p<.018$). Figure 23 presents the work to yield data graphically.

Table 4. The parameter measurements of the bovine lamellar interface after sham or genipin crosslink treatment. Units are as follows: forces are in Newtons, force per width in N/mm, work per width in J/mm, stiffness in N/mm/mm, and stretch in mm/mm.

Parameter	sham mean	sham sd	genipin mean	genipin sd	Pd. Dif. mean	Pd. Dif. sd	Pd. Dif. p value
Yield Force	14.9	9.25	19.7	5.55	7.17	5.70	**0.028**
Yield Force per Width	2.43	1.23	3.87	1.01	1.81	0.87	**0.018**
Stretch at Yield	1.21	0.09	1.23	0.13	0.07	0.12	0.270
Work to Yield per Width	2.16	1.69	3.65	2.67	2.38	1.97	**0.018**
Peak Force	16.2	9.56	22.9	6.80	9.20	5.05	**0.018**
Peak Force per Width	2.65	1.23	4.50	1.01	2.23	0.81	**0.018**
Stretch at Peak	1.26	0.15	1.27	0.21	0.04	0.27	0.734
Work to Peak per Width	3.09	1.43	5.78	5.79	4.32	5.75	0.128
Linear Region Stiffness	1.46	0.23	2.30	1.26	0.71	1.18	0.176

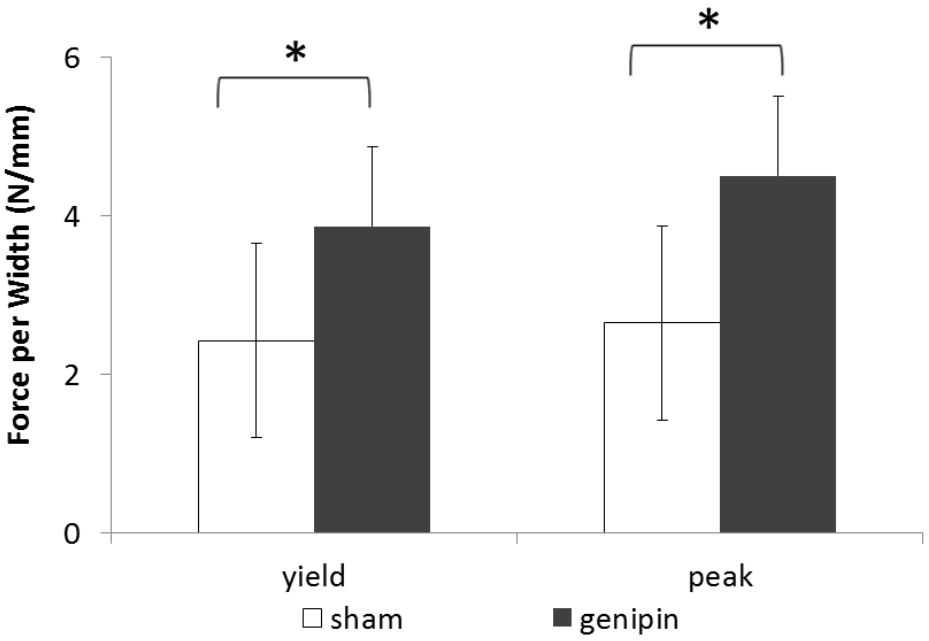

Figure 22. Interlamellar interface force per width. * Denotes statistically significant difference ($p<0.05$) of the paired differences.

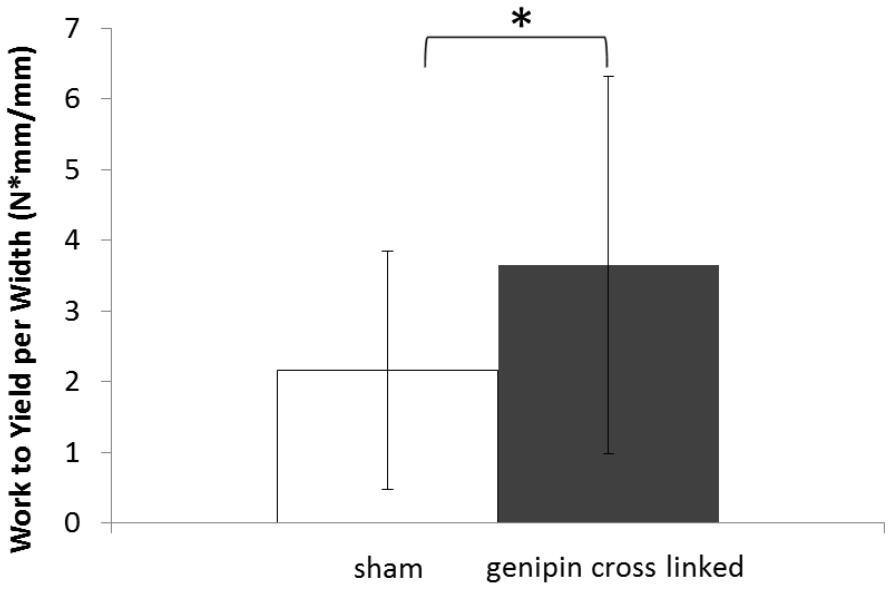

Figure 23. Interlamellar interface work to yield. * Denotes statistically significant difference ($p<0.05$) of the paired differences

5.4. Discussion

The purpose of this study was to determine if crosslinking treatment of the annulus fibrosis would increase the load bearing capability of the interlamellar interface. Specifically, we anticipated changes in the force and work needed to produce slip between the lamella and in the stiffness of the connection. The significant increases in yield force per width, peak force per width, and work to yield after genipin treatment supports our hypothesis that crosslinking increases the load bearing capability of the inter lamellar interface. Additionally, the crosslink treated samples demonstrated a larger mean stiffness than the control samples, but the observed difference was not statistically significant.

The current study results demonstrate some marked differences in the sham treated samples compared to Gregory's initial report[47]. First, Gregory's reported 'load at peak', which according to their description, is more closely associated to a yield force than a peak force, was 0.3 N/mm, approximately 8 times smaller than the 2.43 N/mm observed in control specimens in this study. Gregory's reported mean modulus value was 0.21 N/mm^2 nearly 7x smaller than the 1.46 N/mm^2 of control specimens in this data set. Finally, we were not able to consistently identify a consistent plateau force described by Gregory, but most specimens did demonstrate the ability to carry load even after reaching peak.

There are many differences between this study and Gregory's that preclude direct comparison of the results including the difference in species, preloading, specimen hydration, and geometry. Gregory's study used porcine cervical discs where we used bovine tail discs. Gregory applied a 1400N axial compressive load for 2hr on the intact motion segments, which this study did not duplicate. Such loading may have expelled water from the disc annulus, which would increase the difference in hydration compared to our soaked annulus samples. In this study, we soaked the samples during treatment to provide uniform delivery of sufficient genipin to highly crosslink the tissue. Since the treatment delivery was achieved with a soak, the soak to assist with identification and dissection of the lamella and lamella interface was also performed. Because hydration has been closely linked with the mechanical behavior of the annulus fibrosis [3,46,52,63], the expected large difference in hydration between Gregory's samples and those in this study help to at least partially explain the differences observed between the studies.

Another major difference between the studies has to do with the total number of lamella present on either side of the interlamellar interface. Gregory stated that their specimens consisted of "two intact adjacent AF layers with single-layer tabular ends" and in Gregory's thesis, similar experiments are described as containing two adjacent lamella approximately 0.36mm thick. Our specimens, in contrast, had on the order of 2 to 10 *extra* lamella present on either side. Initially, the extra material was expected to provide additional strength for clamping, but not affect the interacial shear strength. In light of recent works by Schollum[105] and Pezowicz[99] describing a network of fibers that bridge

lamella, it is likely that our specimens, with the extra lamella, may have demonstrated greater resistance to separation due to the inter connectivity of the bridging fibers into the adjacent lamella. Future testing of the lamella connections should take into account the interconnectivity of these bridging fibers for proper interpretation of results with implications for in vivo applications.

The dissection techniques used to achieve a single inter lamella interface were difficult. A detailed dissection protocol was used to achieve a single lamella interface. The dissected half-disc specimens were first allowed to free swell in PBS to enlarge the lamella. After free swelling the specimens were placed on a glass slide and frozen. While frozen, the lamella interface to be tested was identified with the aid of oblique lighting, then using a sharp scalpel the lamella was carefully split on either side of the intended interface allowing the blade to follow the path between the lamella. It was noted in early attempts to dissect specimens that as the width and length of the interface increased, it was more likely that the dissected specimen would contain a lamella that bridged between both clamp sites. Thus the overall length and width of the interface were kept small and the edges of the interface were carefully inspected to ensure a clean cut along the lamella interface plane.

To further minimize the risk of a specimen containing a bridging lamella, specimens were also examined immediately before testing and were further trimmed if a bridging lamella was suspected. During testing, the behavior of the specimens was monitored for

any signs consistent with bridging fibers, especially lack of sliding in the interlamellar interface, very large force to failure, failure of the specimen at the clamping sites before sliding was observed in the interlamellar interface, or asymmetric deformation of the specimen during testing. Three specimens that appeared to be properly dissected but did not fail in the interlamellar interface were discarded from the results. The remaining twelve specimens were judged to be good samples of the interlamellar interface mechanics.

5.5. Conclusion

In conclusion, this study has demonstrated that genipin crosslinking of the annulus fibrosis increases the load carrying capability of the inter-lamellar interface. Significant increases were noted in yield force per width, peak force per width, and resilience (work to yield) associated with the disruption of adjacent lamella after crosslinking treatment. Additionally, a non-statistically significant trend was observed for increased stiffness with crosslinking treatment. Future testing should take into account the possible effects of bridging fibers through adjacent lamella to better characterize the inter lamella interface properties. For example, it may be beneficial to characterize interface strength as a function of the number of adjacent lamella or as a function of bridging fiber density. These results may have implications for an injectable exogenous crosslinking approach to treatment of intervertebral discs to increase the resistance to the initiation and propagation of concentric tears.

6. SUMMARY AND CONCLUSIONS

6.1. Effects of Various Crosslinkers on Tensile Properties of the Disc Annulus

Using different crosslinking agents in individually optimized formulations, differences in the circumferential tensile test properties of the disc annulus were observed. Overall the trend was for the tensile parameters to increase after crosslinking treatment and the changes were unique to the formulations applied.

N-Ethyl-N'-(3-dimethylaminopropyl carbodiimide), or EDC, produced the largest changes (over 300%) of any crosslinker. However, while there where very large changes observed in yield stress and resilience for EDC there was a proportionately smaller change in stiffness. Glutaraldehyde, one of the more commonly reported crosslinkers, demonstrated an increase in stiffness, but a decrease in the other measured parameters relative to control samples. Genipin produced more uniform changes with yield stress, yield strain, resilience, and peak modulus all changing in the range of 123% to 166%. Combined with a relatively lower cytotoxity, genipin appears to be a promising agent for affecting uniform increases in tensile mechanical properties.

6.2. Dose Response Relationship Between Disc Neutral Zone Stability and Genipin Injection Concentration

Bilateral injections into the disc annulus with increasing concentrations of genipin resulted in corresponding changes in joint stability. This data supports that a targeted genipin injection into the disc annulus can effectively alter its neutral zone stability.

At every effective concentration, the effect to neutral zone stiffness was relatively greater than the effect to neutral zone length or ROM. Injections of 20mM genipin did not result in a significant increase in stability for any of the metrics. While 40mM injections produced significant changes in neutral zone stiffness and instability score, 80mM injections were needed to produce significant changes to neutral zone length and ROM. While the highest genipin concentration produced significant decreases in neutral zone length and increase in neutral zone stiffness, the changes were appreciably less than changes from soaking as reported by others, suggesting that further improvements in reagent delivery may be necessary to achieve the changes noted with soaking.

6.3. Resistance of Genipin Crosslinked Annulus Fibrosus to Degradation from Biochemical and Mechanical Loading

Effects to the mechanical behavior of bovine disc annulus after biochemical degradation, cyclic mechanical compression, and crosslinking treatment with genipin were studied. This study supports the hypothesis that genipin crosslinking of the annulus fibrosus

increases the resistance of the tissue to biochemical and mechanically induced degradation.

Accelerated cyclic loading for 10,000 cycles with and without biochemical treatments produced large changes in specimen height and compliance along with visual observations of tissue degradation. Cyclic loading with trypsin was observed to amplify the height loss and compliance increase as well as increase the visually observed decrease in tissue structure. With genipin crosslinking, the effect of the cyclic loading was attenuated so that height loss and compliance increase were less severe and the visual observed tissue structure was maintained.

Both height and compliance of control samples (mechanically cycled but not biochemically treated) recovered after unloaded, overnight soaking. Trypsin height recovered relatively more than the control specimens and compliance remained relatively lower, consistent with matrix degradation but neither effect was significantly different. Recovery of genipin specimen height and compliance was incomplete compared with controls, possibly by a continuation of crosslinking while compacted.

6.4. Effects of Genipin Crosslinking on the Interfacial Shear Resistance of Disc Annulus

The purpose of this study was to determine if crosslinking treatment of the annulus fibrosus would increase the load bearing capability of the interlamellar interface.

Significant increases were noted in yield force per width, peak force per width, and resilience (work to yield) associated with the disruption of adjacent lamellae after crosslinking treatment. These observations support the hypothesis that crosslinking increases the interlamellar shear resistance of annulus fibrosus lamella.

A possible dependency may exist between the observed shear properties and the total number of lamella present on either side of the inter-lamellar interface. In light of recent works describing a network of fibers that bridge lamellae, it is likely that the current study specimens, with extra adjacent lamellae, may have demonstrated greater resistance to shear due to the inter connectivity by the bridging fibers into the adjacent lamellae. Future testing should take into account the possible effects of bridging fibers or the bridging fiber density to better characterize the inter-lamellar interface properties especially for in vivo application.

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